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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : CAROLINE S. BROWN
Serial No. : 07/838,715 Examiner M. Tuscan
Filed : May 4, 1992 Art Unit 1813
For : HUMAN PARVOVIRUS B19 PROTEINS AND
VIRUS-LIKE PARTICLES, THEIR PRODUCTION,
AND THEIR USE IN DIAGNOSTIC ASSAYS AND
VACCINES

August 12, 1993

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

C O M M U N I C A T I O N

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This Communication is supplemental to the amendment filed June 21, 1993 in the above-identified application. In the amendment on pages 6 and 7 applicants mention that copies of certain publications would be provided the Examiner in further support of applicants' invention. Specifically, it was mentioned that the Examiner would be provided with a copy of the publication G. J. Kurtzman et al J. Clin. Invest. 84, 1114-1123 (1988) showing and demonstrating that antibodies raised against the B19 capsid proteins provide protection against B19 infections. A copy of this publication is forwarded herewith as EXHIBIT A.

S/N 07/838,715

Applicants also mentioned that the Examiner would be provided with a copy of the Brown et al publication J. Virol. 66, 6989-6996 (1992). This publication was identified to the Examiner as providing data showing that B19 viruses and VP2/VP1 particles for antigenic determinants and that naturally occurring antibodies bind to the recombinant particles. A copy of this Brown et al publication is forwarded herewith as EXHIBIT B.

Further, on page 7 of the amendment applicants mentioned that animal tests with recombinant VP2 particles having an insertion of epitopes or other pathogens have been carried out and that these tests revealed that such particles (VP2) with a foreign epitope inserted therein are immunogenic against challenge infection. This soon to be published article of Brown et al is entitled "Chimeric parvovirus B19 capsids for the presentation of foreign epitopes". However, a copy of this article now in preparation for publication is being forwarded herewith as EXHIBIT C.

It is respectfully requested that the Examiner, having in mind the amendment filed in this application on June 21, 1993 and the arguments submitted therewith and having in mind the subject communication with accompanying copy of the above-referred publications, should now find the claims presented in this application to be allowable and patentable over the prior art.

S/N 07/838,715

Therefore, favorable consideration and allowance of all the claims pending in this application are respectfully solicited.

I hereby certify that this paper is being deposited this date with the U.S. Postal Service in first class mail addressed to Commissioner of Patents and Trademarks, Washington, D.C. 20231

Thomas F Moran Aug 12 1993
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Respectfully submitted,

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Exhibit A

Vaccines92

MODERN APPROACHES TO NEW VACCINES INCLUDING PREVENTION OF AIDS

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Immune Response to B19 Parvovirus and an Antibody Defect in Persistent Viral Infection

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Abstract

B19 parvovirus has been shown to persist in some immunocompromised patients, and treatment with specific antibodies can lead to decreased quantities of circulating virus and hematologic improvement. A defective immune response to B19 parvovirus in these patients was shown by comparison of results using a capture RIA and immunoblotting. In normal individuals, examination of paired sera showed that the dominant humoral immune response during early convalescence was to the virus major capsid protein (58 kD) and during late convalescence to the minor capsid species (83 kD). In patients with persistent parvovirus infection, variable titers against intact particles were detected by RIA, but the sera from these patients had minimal or no IgG to capsid proteins determined by Western analysis. Competition experiments suggested that this discrepancy was not explicable on the basis of immune complex formation alone and that these patients may have a qualitative abnormality in antibody binding to virus. In neutralization experiments, in which erythroid colony formation in vitro was used as an assay of parvovirus activity, sera from patients with poor reactivity on immunoblotting were also inadequate in inhibiting viral infectivity. A cellular response to purified B19 parvovirus could not be demonstrated using proliferation assays and PBMC from individuals with serologic evidence of exposure to virus. These results suggest that production of neutralizing antibody to capsid protein plays a major role in limiting parvovirus infection in man.

Introduction

B19 parvovirus infection in man is usually acute (1, 2). The only identified target cell for this virus is an erythroid progenitor of the bone marrow (3, 4). Infection in normal individuals is manifest as fifth disease, in which the formation of complexes of antibody and virus is associated with a cutaneous eruption and polyarthralgia (5). In normal persons, infection leads to reticulocytopenia with viremia shortly after inocula-

A portion of this work was included in an abstract published in *Blood*. 1988. 72(Suppl. 1):45a. (Abstr.)

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tion, but the decline in hemoglobin is minimal because of the brief interruption of erythropoiesis (5). In contrast, in individuals with an increased requirement for erythrocytes and heightened marrow erythropoiesis (hemolysis, ineffective erythropoiesis, blood loss, iron deficiency), B19 parvovirus causes transient aplastic crisis, in which the dominant clinical feature is abrupt worsening of anemia (2). In these infected individuals, the period of viremia is short, and the appearance of virus-specific antibody is followed by brisk marrow recovery.

We have identified patients with persistent B19 parvoviremia that has resulted in chronic bone marrow failure. The first reported case was a patient with Nezelof's syndrome (combined immunodeficiency with immunoglobulins); virus was detected in this child's blood for > 18 mo and hemoglobin and neutrophil number were inversely related to viral titer (6). Another patient with a similarly limited clinical immunologic deficit has been studied by us, in whom parvovirus has been persistent for at least 3 and possibly > 10 yr, also resulting in chronic severe anemia (7). Persistent B19 parvovirus infection also may be particularly common among children receiving immunosuppressive chemotherapy for cancer, as the peak incidence of B19 parvovirus infection is in children. Several children with acute lymphocytic leukemia in remission and on maintenance chemotherapy have had chronic anemia and repeatedly viremic sera (8). Finally, we have detected virus in the sera of patients with AIDS who presented with the clinical syndrome of pure red cell aplasia (Frickhofen, N., G. Kurtzman, and N. S. Young, unpublished data).

The host immune response to viruses is complex, and some viruses predominantly elicit either a cellular or humoral immune response (9). Even when neutralizing antibodies or a specific cellular response can be demonstrated in vitro, the protective role of the humoral or cellular arms of the immune system is often inferred from clinical studies of susceptibility in immunosuppressed patients or the results of vaccination. The effectiveness of passive immunization with immunoglobulin (hepatitis A, cytomegalovirus) or hyperimmune serum (hepatitis B, varicella) implies a primary role of antibody in protection against some virus infections. To begin to elucidate the immune response to B19 parvovirus infection, we have studied the antibody and cellular responses to infection in acute and chronic disease.

Methods

Patients. Blood from six patients with persistent B19 parvoviremia was studied (Table I). The first case, T.W. (6) at presentation was a 3½-yr-old boy with combined immunodeficiency with immunoglobulins (Nezelof's syndrome). He became abruptly anemic and granulocytopenic at age 1½ yr, when his serum for the first time contained virus: he has been intermittently viremic and transfusion-dependent for more

Table I. Summary of Results of Anti-B19 Parvovirus Antibody Testing

Patient	Diagnosis	Date	IgM	IgG	Immunoblot	Virus
T.W.	CID	21-IV-86	19	32	Neg	+
		5-IX-86	72	19	Neg	-
		4-XI-86	31	20	Neg	+
E.Fo.	CID	7-III-88	>100	17	Pos	+
		25-V-88	>100	24	Pos	+
L.G.	ALL	28-XII-87	<1	<1	Neg	+
		4-II-88	>100	28	Pos	-
		8-III-88	9	14	Pos	-
		21-III-88	2	5	Pos	+
		21-IV-88	>100	39	Pos	-
E.Fr.	ALL	22-XI-87	<1	<1	Neg	+
		4-II-88	84	8	Neg	±
		31-III-88	12	17	Pos	+
		17-V-88	56	46	Pos	-
C.L.	AIDS	24-X-86	<1	<1	Neg	+
		14-X-87	39	37	Neg	-
G.F.	AIDS	22-XII-87	<1	<1	Neg	+

IgG and IgM were determined by capture RIA and results expressed in arbitrary units. Virus was determined by dot-blot analysis. ALL, acute lymphocytic leukemia; CID, congenital immunodeficiency.

than 2 yr. He has no other symptoms suggesting a viral infection, and his only other manifestation of underlying immunodeficiency was an episode of culture-negative pneumonia at age 6 mo. Another patient, E.Fo. (7), is a 24-yr-old man who has been parvoviremic for at least 3 and possibly > 10 yr, when he and his brother simultaneously and abruptly developed pure red cell aplasia requiring erythrocyte transfusion support. This patient has a presumed diagnosis of underlying immunodeficiency; although he has not had opportunistic infections other than B19 parvovirus, detailed evaluation has shown anergy, suboptimal response of PBMC to a test panel of antigens and mitogens, and low serum IgG levels. Two patients, E.Fr. and L.G., developed chronic parvovirus infection in the setting of acute lymphocytic leukemia in remission and on maintenance chemotherapy (8). Finally, two patients with AIDS secondary to HIV infection had concurrent parvovirus infection. C.L., a 24-yr-old male homosexual with antibodies to HIV, presented with pure red cell aplasia (10). With corticosteroid therapy, he developed *Pneumocystis carinii* pneumonitis. He later showed dramatic hematologic recovery after treatment with high-dose immunoglobulins. Finally, G.F. was a 27-yr-old male intravenous drug abuser who presented with septic arthritis and severe anemia. His blood showed the presence of parvovirus, antibodies to HIV, and *Staphylococcus aureus*; he died 5 d after admission of sepsis.

All infected patients have been characterized as having intermediate levels of parvoviremia by DNA dot blot analysis (10^5 – 10^{10} viral genome copies/ μ l serum) and in some (T.W., E.Fr., G.F.) viral replication forms have been demonstrated by Southern analysis of bone marrow cells. The bone aspirate smears in all cases showed absence of erythroid precursors and the presence of giant pronormoblasts.

Control blood samples were obtained from patients with acute parvovirus infection, manifested as transient aplastic crisis or fifth disease, and normal laboratory personnel with serologic evidence of past parvovirus infection.

Detection of serum B19 virus and antibody. Viral DNA was detected by extraction of DNA from 10 μ l of sera (11) and in some cases

cells (12) and dot blot hybridization with a ^{32}P -nick-translated full-length B19 probe (pYT103; 13). Serum was analyzed for viral antigen and anti-B19 virus specific IgM and IgG by RIAs (14). Electron microscopy was performed on pellets after ultracentrifugation of 50 μ l of serum; negative staining was done using 3% phosphotungstic acid, pH 6.5.

Immunoblot. Virus from the serum of a patient with transient aplastic crisis was separated from other serum components by centrifugation through 40% sucrose at $\sim 100,000$ g for 6–16 h (4). Virus was resuspended in PBS and, after heating to 90–100°C for 10 min under reducing conditions, electrophoresed in 8% SDS-polyacrylamide (15). After electrophoretic transfer to nitrocellulose, individual lanes were incubated with separate sera in a Deca-Probe apparatus (Hoefer Scientific Instruments, San Francisco, CA) for 1 h at room temperature. IgG was detected by incubation of the filter with ^{125}I -Protein A or ^{125}I -goat anti-human IgG (New England Nuclear, Boston, MA) and IgM by incubation with rabbit anti-human IgM (Miles Laboratories, Naperville, IL) followed by ^{125}I -goat anti-rabbit IgG (New England Nuclear) by the nonfat dry milk-antifoam-thimerosal (16) method.

Detection of neutralizing antibody. Sera from the patient were tested for their ability to abrogate the cytotoxic effect of B19 virus in erythroid colony formation assays (3, 4). Appropriate controls were selected with approximately equivalent quantities of anti-B19 specific IgG and IgM determined by RIA. 75 μ l of heat-inactivated serum or dilutions were incubated with B19-containing serum at 4°C overnight and then serum mixes were incubated with fresh bone marrow mononuclear cells for 4 h at 4°C. Erythroid colony-forming cell- (CFU-E)¹ derived colony formation was assayed after incubation of cells in semisolid media at 37°C, 5% CO₂, 95% humidity for 7–8 d.

Cell proliferation assay. PBMC from heparinized blood were prepared by Ficoll-Hypaque density gradient sedimentation and washed twice in RPMI 1640 medium with 2% autologous serum or FCS. Cells were cultured at a concentration of $2\text{--}3 \times 10^6$ /ml in 150 μ l total volume in 96-well microtiter plates in RPMI 1640 medium containing either 15% autologous serum or FCS with supplemental glutamine, penicillin, and streptomycin; cultures remained at 37°C and 95% CO₂ until harvested at days 2, 4, or 6. Test mitogens and antigens included PHA, Con A, PWM, candida, tetanus toxoid, streptolysin O, and dilutions of partially purified B19 parvovirus or empty parvovirus capsids. Virus was purified by passage of serum over a 40% sucrose cushion at 38,500 rpm \times 16 h and suspension of the pellet in ~ 100 μ l of PBS; samples from uninfected sera were prepared similarly. Virus content was estimated by dot-blot analysis with a radioactively labeled virus-specific DNA probe. The serum used (Gray) contained only virus and was free of immune complexes as determined by electron microscopy. In addition, a lysate of a genetically engineered cell line that produces empty B19 parvovirus capsids (17) was also used as an antigen in some experiments. Proliferation was determined by counting washed cells after a 4-h incubation with [³H]thymidine (New England Nuclear; 20 Ci/mmol sp act), 1 μ Ci/well.

Results

Normal humoral response to B19 parvovirus infection. B19 parvovirus has two capsid proteins, which are encoded by overlapping transcripts from the right side of the genome (Fig. 1; 18, 19). The major species, termed VP2, is 58 kD and constitutes $\sim 90\%$ of the capsid protein (18); the minor species (VP1) is 83 kD and includes 227 amino acids at the amino terminus not present in the major capsid protein species. The

1. Abbreviations used in this paper: CFU-E, erythroid colony-forming cells.

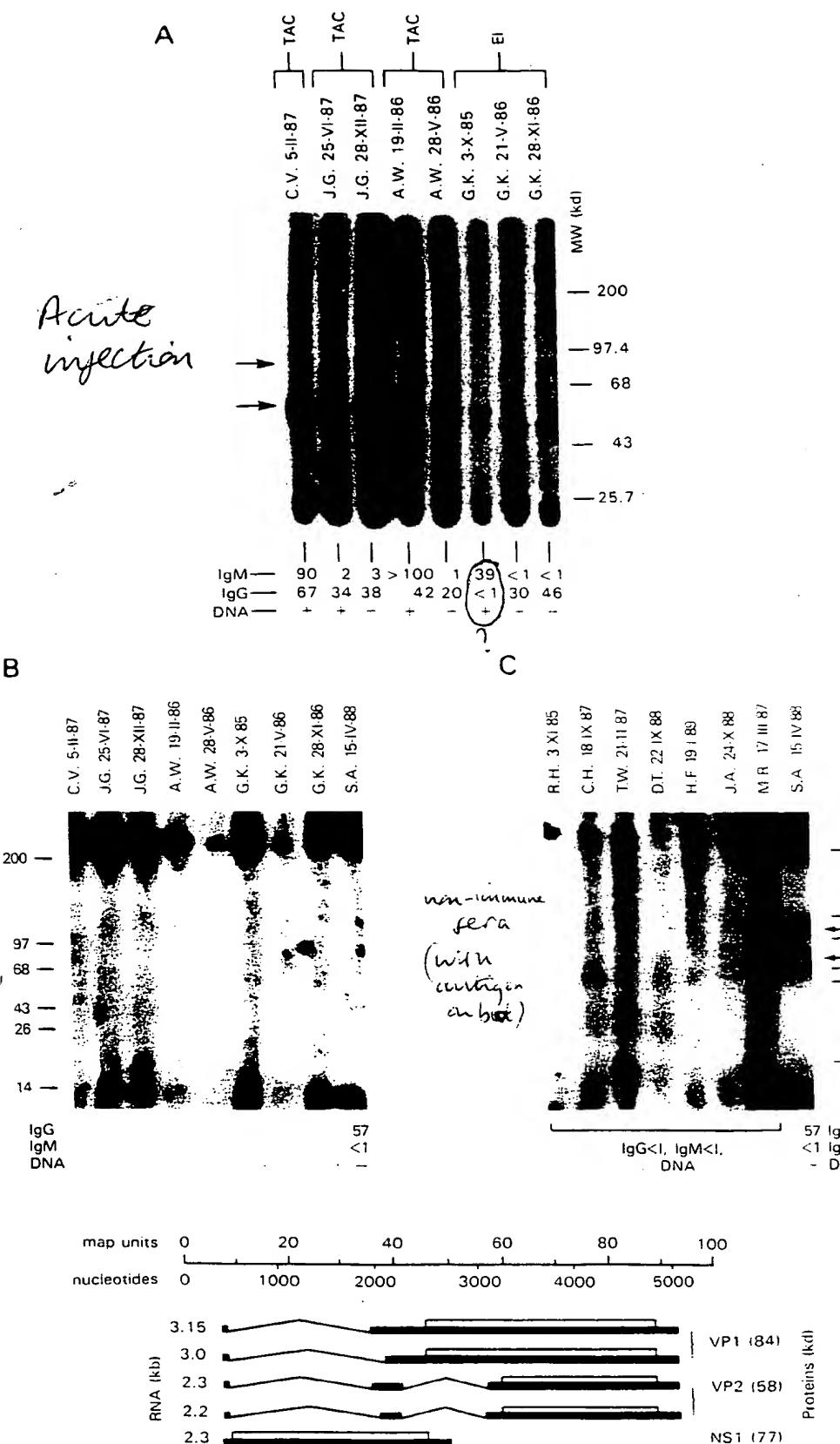


Figure 1. Immunoblot of sera from patients with a history of acute B19 parvovirus infection and nonimmune controls. Serum containing B19 virus or nonviremic serum was electrophoresed in 8% SDS-PAGE under denaturing conditions, and after transfer to nitrocellulose each lane was exposed to a different serum sample: all sera were tested at a dilution of 1:50. Antibody binding was detected by autoradiography after incubation with ^{125}I -goat anti-human IgG. Arrows, position of the 58- and 83-kD capsid proteins. Shown below each lane are titers of virus-specific IgG and IgM determined by capture RIA (expressed in arbitrary units) and the presence (+) or absence (-) of B19 DNA in serum or peripheral blood leucocytes determined by dot blot hybridization for the same blood samples. (A) Immunoblot of sera from patients with acute parvovirus infection with B19 antigen (transient aplastic crisis [TAC] in hemolytic disease, erythema infectiosum [EI] or fifth disease in a normal person). In three cases, paired samples were obtained early, on diagnosis of infection, and during the late convalescent phase; (B) immunoblot of the same sera using nonviremic serum as antigen; (C) immunoblot of nonimmune sera from hematologically normal donors and hematologic controls with a history of frequent transfusions using B19 parvovirus as antigen. Beneath the autoradiograph is a reference diagram of a simplified version of the genomic organization of the B19 parvovirus: note the overlap of the transcripts for the major and minor capsid proteins and the small region unique to the VP1 sequence.

capsid proteins predicted from analysis of the B19 genomic sequence have been detected using either convalescent human serum after in vitro translation (19) or by rabbit polyclonal serum directed against fusion proteins, which were produced in genetically altered bacteria that carry a portion of the capsid protein gene (20).

We examined 15 sera from patients in whom B19 parvovirus infection had been established by detection of antibodies in a capture radioimmunoassay; on Western analysis, antibody specificities for either the 58-kD capsid protein, the 83-kD capsid protein, or both species were always present. Examination of paired specimens from early and late during the convalescent phase showed a consistent pattern of protein recognition (Fig. 1 A). At the time of viremia, IgG antibody to virus was often not detected by immunoblot; however, when it was detected, sera reacted only with the major capsid protein of 58 kD. During early convalescence (< 1 wk from viremia), antibody to the major capsid protein was the predominant or exclusive reactivity. Weeks to months after infection or in sera from an immune population routinely screened, reactivity to the 83-kD protein was present and often dominant. Because

much less of the 83-kD protein is present in virus and therefore on the nitrocellulose used for immunoblotting, the Western method probably underestimated the amount of 83-kD specific antibody in serum. In addition, failure to detect a major capsid protein reaction indicated that epitopes present at the amino-terminal end of the 83 kD, which are not shared between the two capsid proteins, are specifically recognized. There was no reaction of immune sera to a mock preparation of antigen from a nonviremic individual (Fig. 1 B), nor did nonimmune sera from normal individuals or frequently transfused patients with hematologic diseases bind to parvovirus capsid proteins (Fig. 1 C).

Commercial immunoglobulin preparations also contained anti-B19 parvovirus antibodies. All of seven lots (six Gammimmune, Cutter, Elkhart, IN; one Gammagard, Hyland, Glendale, CA) contained antibodies determined by RIA, and four of four lots showed specificities to both 58- and 83-kD capsid proteins by immunoblot (Fig. 2).

Antibody response to B19 parvovirus in persistent infection. In the first case of persistent infection, both IgM and IgG antibodies specific for B19 parvovirus were readily detected by

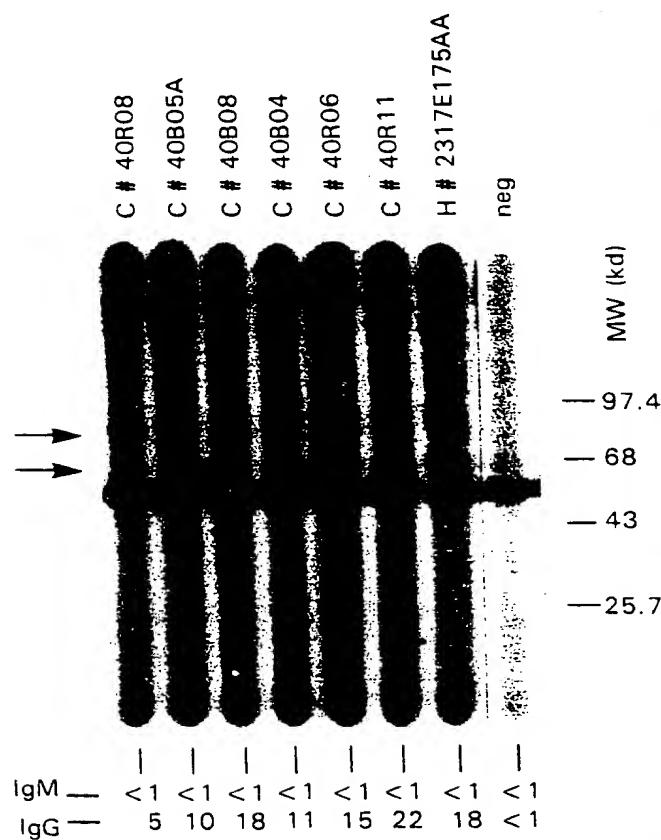


Figure 2. Immunoblot of commercial preparations of immunoglobulin. Six lots of Gammimmune-N (Cutter Biologicals; abbreviated as C with lot number) and one lot of Gammagard (Hyland Laboratories; abbreviated H with lot number) were assayed at 1:50 dilutions as described in the legend to Fig. 1. Arrows indicate the position of the 58- and 83-kD major and minor capsid proteins. The dense band migrating at 55 kD represents the heavy chain of IgG. No immunoglobulin was added to the lane labeled "neg."

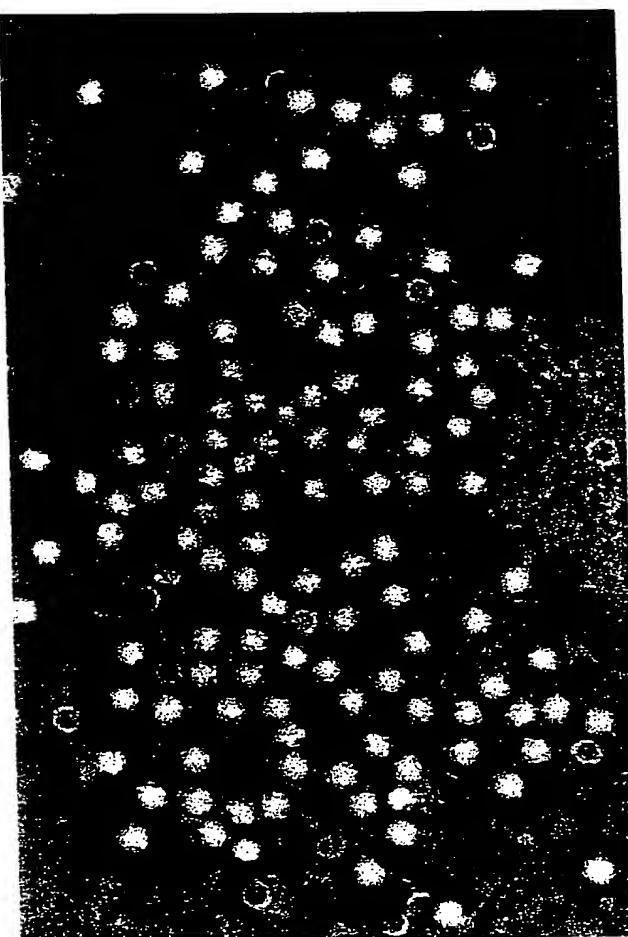


Figure 3. Electron micrograph of parvovirus in patient T.W.'s serum (from 23.VII.86). Viral particles were visible after sedimentation, without added anti-B19 parvovirus serum because of aggregation by patient's antibody, mostly IgM (negative stain, $\times 173,000$, Bar. 100 nm).

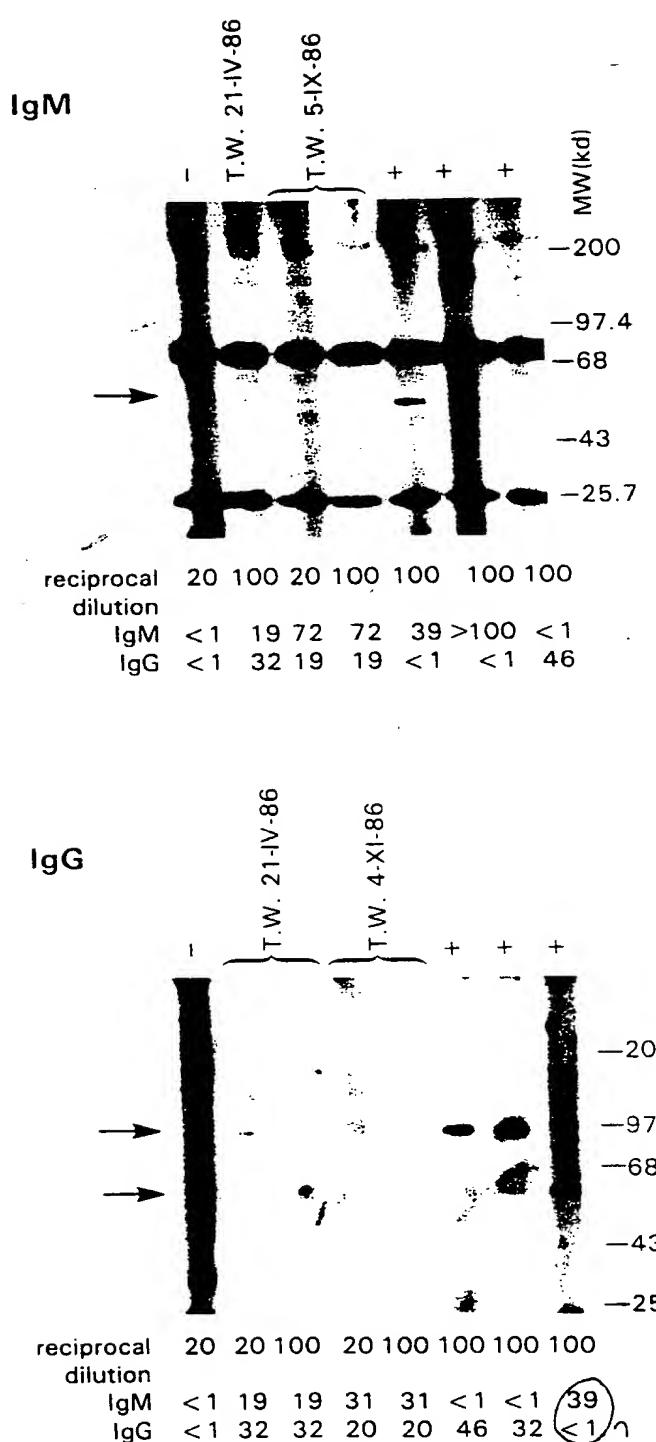


Figure 4. Immunoblot of sera from patient T.W. with congenital immunodeficiency and persistent parvovirus infection. Experiments were performed as described in the legend to Fig. 1, except that anti-parvovirus IgM (A) was detected using rabbit anti-human IgM followed by ^{125}I -goat antirabbit IgG, and anti-parvovirus IgG (B) was detected using ^{125}I -protein A. Arrows show the position of capsid protein bands of 83 and 58 kDa. IgM, which contaminates the virus pellet, is apparent as multiple bands in A. The reciprocal of the dilution of serum reacted and the titer of antibody by RIA are shown

a capture RIA. In addition, IgM antibodies apparently cross-linking virus particles were visualized by electron microscopy of centrifuged sera from the patient (Fig. 3). However, multiple serum samples failed to react with electrophoresed capsid proteins on immunoblotting for B19 parvovirus-specific IgG and IgM (Fig. 4). (Apparent slight reactivity in one sample only was probably due to recent transfusion with immune plasma from the patient's father.)

To determine whether the absence of anticapsid protein specificity in serum on immunoblot was a consistent feature of persistent infection, we examined sera from other patients with chronic B19 parvoviremia (Fig. 5). Persistent parvovirus infection has been documented in children with acute lymphocytic leukemia in remission receiving immunosuppressive chemotherapy. Sera obtained from these children during periods of viremia and anemia failed to react with capsid proteins on immunoblotting and also had low titers of virus-specific IgG and IgM on RIA, whereas sera from periods in which virus temporarily disappeared from the circulation and at ultimate recovery did react on immunoblot (Table I). In a patient with AIDS who clinically presented with pure red cell aplasia, antibody initially was lacking by both tests. He later recovered erythropoiesis with immunoglobulin therapy (10). A serum sample obtained 1 yr later showed high antibody titers by RIA but no reactivity on Western analysis. As summarized in Table I, no reaction or, in one case (E.Fo.), a weak reaction on immunoblotting was a consistent feature of the immune response in patients who developed virus persistence. Reactivity of sera from patient E.Fo., as well as from the two patients with leukemia after eventual clearance of virus, was only directed against the 58-kD major capsid protein, and these sera consistently failed to detect the 83-kD minor capsid protein.

Discordance between the results of the capture RIA, immune electron microscopy, and immunoblot might have a quantitative or qualitative explanation. As the RIA and electron microscopy also detect immune complexes (14), a quantitative defect in antibody production could result in a deficiency in free antibody for immunoblot detection. Alternatively, patients might produce qualitatively abnormal antibody, capable of binding to conformational determinants of particles in the RIA but not of binding to epitopes of capsid protein as presented on immunoblot. To test these hypotheses, we mixed dilutions of virus with sera containing antibody before immunoblotting. Although some adsorption of binding activity was observed, the amount of virus required to decrease the intensity of the immunoblot reaction was at least 10-fold the highest concentration of virus present in the blood of T.W. and many more orders of magnitude higher than the virus concentration present in E.Fr. or C.L. This result suggested the presence of a qualitative defect in antibody recognition of virus proteins.

Neutralization of virus activity by sera. B19 parvovirus has only been propagated in human erythroid bone marrow cells.

below the autoradiograph. Patient T.W.'s sera on the dates tested contained parvovirus. Positive control samples (+) from patients with a history of parvovirus infection were selected for equivalent titers of IgG and IgM antibody to parvovirus by RIA. The negative control (-) that lacked B19 parvovirus antibody was from a multiply transfused sickle cell anemia patient.

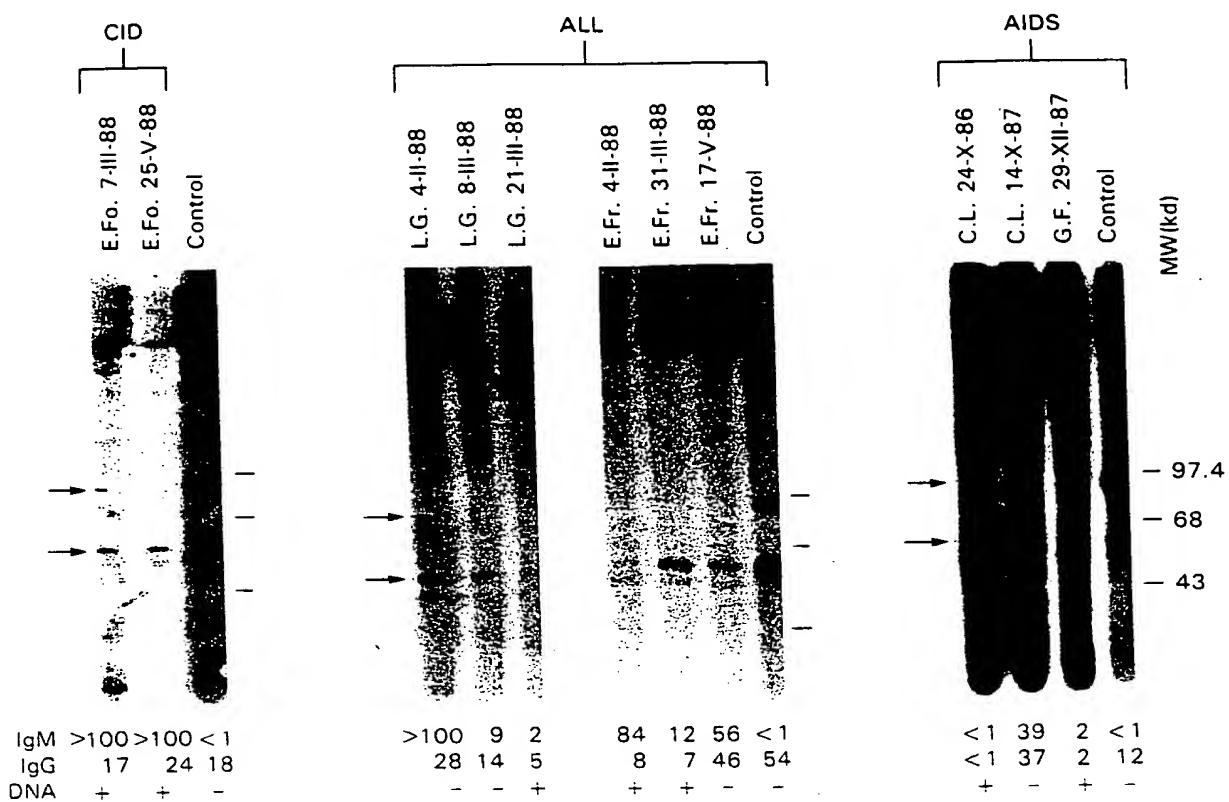


Figure 5. Immunoblot of sera from patients with persistent parvovirus infection. Underlying diagnoses are indicated above the autoradiograph (congenital immunodeficiency [CID], acute lymphocytic leukemia [ALL], AIDS) and the antibody titer by RIA below. The method was as described in the legend to Fig. 1. All patient sera were tested at a dilution of 1:20; the control serum was at 1:50. Arrows show the position of capsid protein bands at 58 and 83 kD.

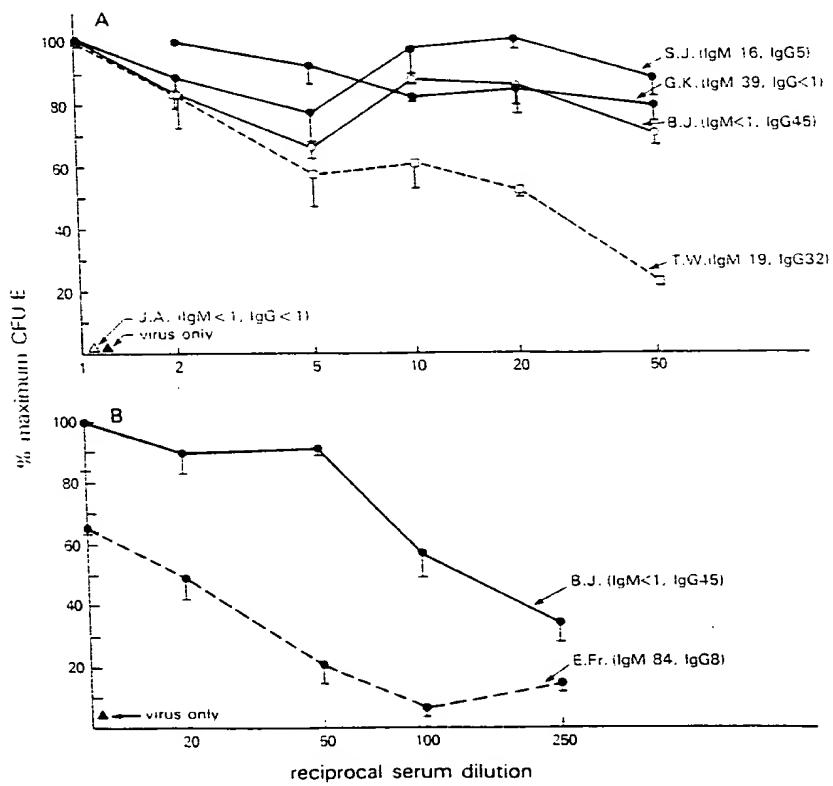


Figure 6. Neutralization of parvovirus activity by sera from persistently infected patients T.W. (A) and E.Fr. (B). Normal sera were selected for equivalent antibody titers by RIA for experiment A. Sera containing antibody to virus were diluted as shown on the abscissa. CFU-E-derived colony formation was almost completely inhibited by the addition of virus alone or by virus mixed with nonimmune serum. Bars represent SEM.

Ability of the virus to inhibit erythroid colony formation provides an assay of virus activity (3, 4). The virus is most cytotoxic at the stage of a late erythroid progenitor cell (CFU-E): the appearance of erythroid colonies at 7–8 d in semisolid medium enriched with erythropoietin is virtually eliminated by preincubation with B19 parvovirus.

We tested sera for their ability to abrogate inhibition of erythroid colony formation by virus, comparing sera from two patients that contained anti-B19 parvovirus antibodies (Fig. 6). Compared with sera from normal persons, who were selected for similar titers of anti-B19 parvovirus IgG or IgM determined by capture RIA, the serum of the child with Nezelof's syndrome was obviously deficient in neutralizing activity (Fig. 6 A). Similarly, the serum from a patient with acute lymphocytic leukemia in remission also had very poor neutralizing activity in comparison to another normal convalescent phase serum (Fig. 6 B). Sera from both persistently infected patients had poor reactivity on Western analysis but measurable titers of antibody by capture RIA.

Although sera from normal exposed individuals consistently neutralized virus, there were differences in potency among sera at dilutions higher than 1:50, and the sera that were most effective at blocking virus infectivity also showed the intensest reaction on immunoblot (Fig. 7). These results suggest that the ability of sera to react with denatured capsid proteins on immunoblot could be approximately correlated to their capacity to neutralize virus infectivity. We similarly compared serum specimens in which reactivity on immunoblot was predominantly directed to the major capsid species (58 kD) or to the minor capsid protein (83 kD) (Fig. 8). The equivalent neutralizing ability of these sera indicated that even serum containing antibody restricted to the unique portion of the minor capsid protein, as develops during convalescence, was fully capable of inhibiting infectivity of virus.

Inability to detect a cellular response to B19 parvovirus. Multiple attempts were made to detect a proliferative response by peripheral blood cells to B19 parvovirus. Using preparations of unfractionated blood mononuclear cells from seropositive normal donors, variable concentrations of purified virus, and different times for harvest of cultures, no increase in incorporation of radioactive thymidine was observed (Fig. 9). Because of the possibility that immune complexes formed with antibodies present in autologous serum might block cell access or inhibit proliferation, experiments were performed in which FCS or parvovirus antibody-negative human serum replaced autologous serum, and empty parvovirus capsids generated from a cell line and serum containing virus served as antigens. Under these conditions, we were again unable to detect a proliferative response to virus antigen at 3–7 d (data not shown).

Discussion

In normal individuals infected, either naturally or experimentally, with B19 parvovirus antibodies to virus form rapidly, usually within a week of inoculation (21). Fifth disease is itself an antibody-mediated, probably immune complex disorder, occurring at the time that antibodies are present in the circulation and seldom associated with the presence of virus in serum (22). (We have observed classic symptoms of fifth disease in immunosuppressed, persistently infected patients following immune plasma [E.Fr.] or immunoglobulin [E.Fo.]

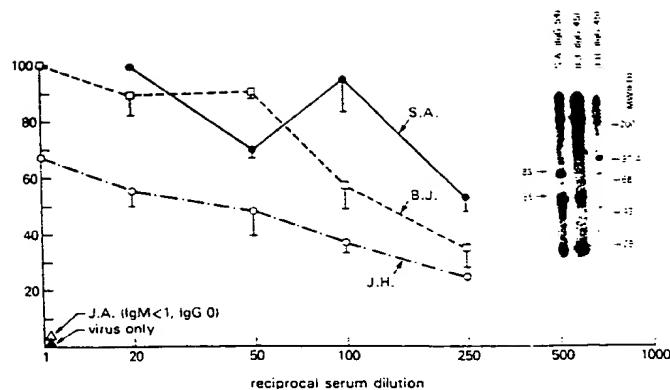


Figure 7. Neutralization of parvovirus activity by sera from normal individuals. Three normal sera were selected based on varying strength of reaction on immunoblot (inset). Bars represent SEM.

therapy.) On immunoblot of normal sera early after infection, antibody specificity to the 58-kD capsid protein predominates: a similar result has been recently reported by others (23). The amino acid sequence of the 58-kD major capsid protein is contained within the 83-kD minor capsid sequence, and antibodies may recognize some unique features of the major capsid protein. Such Mabs with specificity for the major capsid protein of canine parvovirus have been produced (24). Late in convalescence the 83-kD species antibody specificity predominates: these antibodies presumably recognize the unique 227-amino acid region of the larger protein. The topology of the major and minor proteins on the parvovirus capsid surface is not known. The unique region of the 83-kD protein has areas of pronounced hydrophilicity and several predicted alpha helices at the amino terminus, which might contribute to its immunogenicity (25).

Formation of neutralizing antibodies is a consistent feature of acute infection in animals by members of *Parvoviridae* (26). Diluted sera from patients who have been exposed to the virus

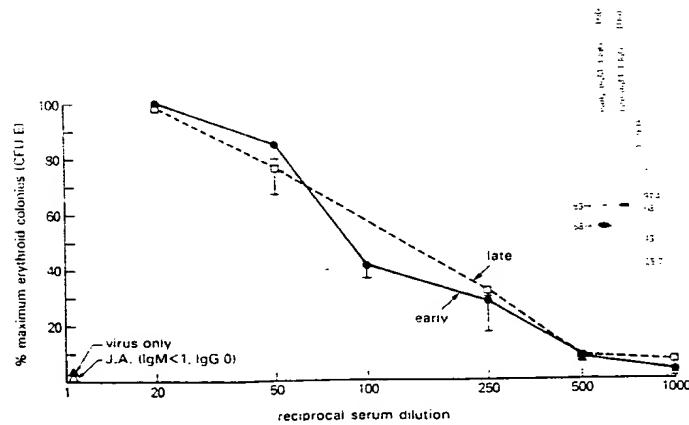


Figure 8. Neutralization of parvovirus activity by normal sera. The ability of sera to block inhibition of erythroid colony formation by virus infection was determined by paired sera from a normal child. Serum obtained in early convalescent phase predominantly bound 58-kD capsid protein, and late convalescent phase serum bound 83-kD protein as shown in the inset immunoblot. Bars represent SEM.

can partially or entirely neutralize the inhibitory effect of virus on erythropoietic colony formation; sera containing either IgM or IgG have virus neutralizing ability (4). Sera that contain antibodies directed predominantly to the 83-kD minor capsid protein specificities were equivalent to sera containing predominantly anti-58-kD major capsid specificities (experiments with diluted sera indicated that this was not an artifact secondary to underestimation of the amount of antibody to the minor capsid species by the immunoblot technique). In contrast, we were unable to demonstrate a proliferative cellular response to B19 parvovirus, using a number of donors and under a variety of experimental conditions. Lymphocytes from some mink infected with Aleutian disease virus also fail to proliferate in vitro on exposure to virus (27-29). Our results would suggest that the humoral response to B19 parvovirus is most important in resolution of disease, a conclusion reinforced by clinical trials.

Persistent infection with B19 parvovirus in humans causes chronic anemia. Persistence has been observed in the fetus (30) and in three populations of immunosuppressed patients: congenital immunodeficiency, acute lymphocytic leukemia receiving chemotherapy, and AIDS. However, none of the patients that we have studied suffered obviously from other opportunistic infections. In other words, chronic parvovirus was the major clinical consequence of their presumed immune deficit, suggesting that in some patients the immune deficit may be restricted, subtle, and only apparent in retrospect. Parvoviruses can infect lymphocytes (31-33), and a strain of the rodent parvovirus minute virus of mice is specifically lymphotropic and immunosuppressive (33). Although it is not possible to exclude rigorously an effect of the human parvovirus on the immune response, we have not been able to demonstrate B19 parvovirus replication in lymphocytes cultured in vitro or the presence of the virus in lymphocytes of persistently infected patients (Kurtzman, G. J., and N. S. Young, unpublished data).

Persistent infection of animal parvoviruses in young hosts is common: virus is shed in feces and urine for long periods after initial inoculation, during either convalescence or in latent infection, in mink (34), cats (35), and pigs (36). The rela-

tionship of persistent animal parvovirus infection to antibody production is complicated. Formation of neutralizing antibodies is believed to have a major role in resolution of acute infection in adult animals (37), but virus-specific antibodies are often present in persistently infected neonatal animals, and abnormally elevated titers of antibody have been associated with latency. Reappearance of clinical illness in animals has followed immunosuppressive therapy. In Aleutian mink disease, which is chronic and often fatal, tissue damage is the result of deposition of immune complexes of parvovirus antigen and antiviral IgG antibodies that fail to neutralize virus (38).

In our experiments, there was discordance between the results of capture RIA and immunoblot in detection of anti-parvovirus antibody. As these patients were viremic, one obvious explanation is that antivirus antibody was present in complex with virus. Such complexes with IgM were visualized by electron microscopy of T.W.'s sera. Antibodies in immune complexes would be predicted to be detected in the capture RIA, as they would bind to plastic coated with anti-human IgG and IgM antibodies, and virus in complexes could be recognized by the antivirus MAb. In the absence of free antibody or antigen binding sites, antivirus antibody in complexes would not be detected in immunoblot. However, control experiments showed that failure to react on immunoblot was not a consequence of immune complex formation. A qualitative antibody defect was further suggested by the presence of some activity in neutralization assays, indicating the availability of free antigen binding sites. These results suggest that some patients (leukemia in remission with chemotherapy) do not produce anti-B19 parvovirus antibody (a quantitative defect) and that other patients (congenital and acquired immunodeficiency) may produce antibodies that fail both to recognize capsid protein epitopes and to neutralize virus activity. An example of a combined quantitative and qualitative defect in antibody production in response to viruses is infection of ferrets with Aleutian disease virus: in animals with low titers of antibody, antibody is directed primarily against conformational virus determinants, whereas ferrets with high titers also produce antibody to sequential determinants detectable on

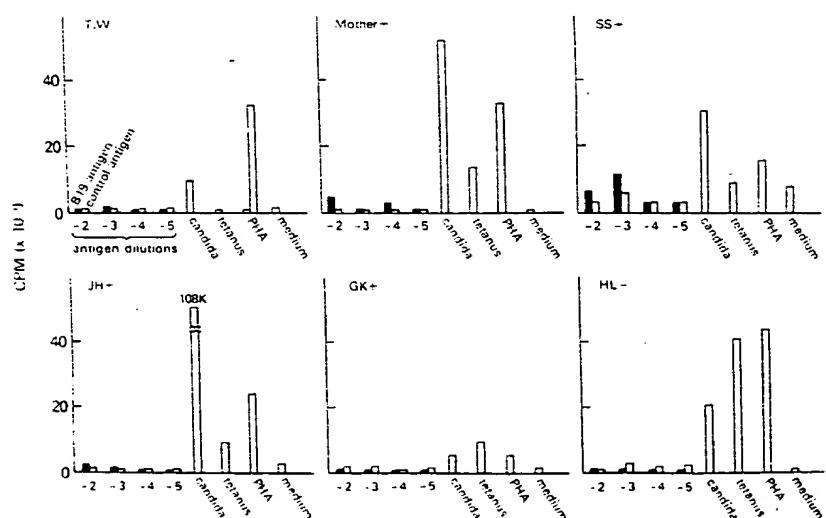


Figure 9. Cellular response to parvovirus. Peripheral blood mononuclear cells from patient T.W. with persistent infection, his mother (with high titers of anti-B19 antibodies by RIA), three individuals with serologic evidence of past parvovirus infection (S.S., J.H., and G.K.) and a seronegative person (H.L.) were assayed for proliferation in response to purified B19 parvovirus at varying concentrations (10^{-2} - 10^{-5} dilutions of a preparation that contained genome 10^{11} genome copies/ml; shaded bars), to normal sera processed as for virus purification (open bars), and to test antigens candida, tetanus, and PHA. Cells incubated in medium without antigen or mitogen served as a negative control (medium). In this experiment, cells were incubated for 7 d with test antigens before addition of [3 H]thymidine; there was also no reactivity to B19 parvovirus at 3 d.

Western analysis (39). In general, animals repeatedly exposed to antigen not only switch from IgM to IgG production but also show progressively higher levels of IgG with increasing affinity and specificity (40). The late production in normal persons of neutralizing IgG that is directed toward more restricted epitopes on the B19 parvovirus minor capsid species may be the result of selection of B cell clones. In patients persistently infected with B19 parvovirus, the prolonged period of elevated antivirus IgM (Table I) as well as the lack of antibody specificity for the 83-kD minor capsid species would also support a class switching defect.

The major immune response to B19 parvovirus appears to be humoral. Based on these data, we have treated several persistently infected patients with immunoglobulin preparations. T.W. received both his father's plasma and commercial immunoglobulin; he experienced a transient reticulocytosis to 8% and erythroid precursors appeared in the bone marrow, although the hemoglobin did not rise, possibly due to iatrogenic hemolysis from the high concentrations of IgG infused. E.Fr. underwent a course of treatment with his father's plasma and experienced a brief reticulocyte response as well as severe fifth disease; sufficient plasma could not be administered to sustain antiparvovirus immunoglobulin levels. E.Fo. has received very high doses of commercial IgG and has had a complete hematologic remission, associated with disappearance of virus from the circulation (7). Correction of the antibody deficit in persistently infected patients may allow reversal of their hematologic disease.

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Localization of an Immunodominant Domain on Baculovirus-Produced Parvovirus B19 Capsids: Correlation to a Major Surface Region on the Native Virus Particle

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An immunodominant region on baculovirus-produced parvovirus B19 VP2 capsids was localized between amino acids 259 and 426 by mapping the binding sites of a panel of monoclonal antibodies which recognize determinants on the particles. The binding sites of three monoclonal antibodies were fine-mapped within this antigenic domain. Six VP2-specific monoclonal antibodies recognized determinants common to both the empty capsids and native parvovirus. The defined antigenic region is most probably exposed on the native B19 virion and corresponds to part of the threefold spike on the surface of canine parvovirus particles.

Human parvovirus B19 causes the fifth disease of childhood, a mild infection, and a variety of other illnesses as a result of its almost exclusive replication in erythroid progenitor cells of the bone marrow (21, 38). In individuals with an increased requirement for erythrocytes in combination with hemolysis, parvovirus B19 causes transient aplastic crisis, and in immunocompromised patients, it can cause prolonged anemia (35, 37). Primary infection in pregnancy has been associated with spontaneous abortion and hydrops fetalis (1, 33). The requirement of B19 virus for erythroid progenitor cells has meant that culture of the virus has not been established in a cell line; to obtain antigen for diagnostic use and possibly a vaccine, expression systems have been used. Empty capsids consisting of the two capsid proteins VP1 and VP2 have been produced in Chinese hamster ovary cells (15) and insect cells (4, 14). Expression of VP1 and VP2 independently from two baculovirus recombinants showed that VP2 (the major capsid protein) can alone assemble into empty capsids in insect cells whereas VP1 cannot. The VP2 and VP1/VP2 capsids produced in insect cells are similar to native virus in size, appearance, and stoichiometry in the case of the capsids containing VP1 (4). VP1 differs from VP2 in that it has an N-terminal extension of 227 amino acids which is thought to be internally located (6, 9). In a capture enzyme-linked immunosorbent assay (ELISA) in which the reactivities of the capsids with B19 virus specific antibodies bound to the solid phase were tested and in which a degree of conformation of the capsids is retained, both types of insect cell-produced capsids were recognized by all human sera containing B19 virus-specific antibodies that were tested (23). For the development of a B19 virus vaccine based on the recombinant capsids, it will be necessary to analyze, among other properties, the immunogenicity and epitopes of the capsids, the latter in comparison with those mapped on the native virion. Since it may also be possible to use these recombinant B19 virus capsids as a vaccine

carrier, as has been described for a number of polymeric particulate proteins (reviewed in reference 16), the mapping of epitopes may reveal candidate sites for the insertion of foreign epitopes. To achieve these aims, a panel of monoclonal antibodies (MAbs) was made by immunizing mice with the purified recombinant VP2 capsids. Their binding sites were mapped by using overlapping bacterial fusion proteins spanning the whole of the capsid protein VP1, and fine-mapping was carried out by means of an ELISA with overlapping synthetic nonapeptides (PEPSCAN) spanning the region to which most MAbs were shown to bind. Epitopes recognized by two MAbs raised against native B19 virus were also mapped.

The results presented here demonstrate an important, immunodominant region on the synthetic VP2 capsids which correlates with a similar region found on native B19 virions. Further, three determinants that may represent immunoexposed epitopes could be precisely defined.

MATERIALS AND METHODS

Expression of B19 virus capsid proteins in insect cells. Construction of the recombinant baculoviruses expressing VP1, VP2 (3), and both VP1 and VP2 (4) has been described previously. Baculoviruses were grown in *Spodoptera frugiperda* cells (5), obtained from the American Type Culture Collection (CRL 1711), in TC-100 medium (GIBCO/BRL) containing 10% fetal calf serum and 50 µg of gentamicin per ml. Infections were carried out on monolayers of *S. frugiperda* cells at a multiplicity of infection of 1 PFU per cell, and infected cells were harvested 3 days postinfection. All baculovirus manipulations were performed as described previously (31). Capsids consisting of VP2 were purified from infected insect cells essentially as described previously (4) except that a 28% (wt/wt) CsCl gradient was used as the last purification step. This material was centrifuged to equilibrium at 100,000 × g for 24 h at 5°C. The banded material was dialyzed against three changes of phosphate-buffered saline (PBS), and the protein concentration was determined

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by the Bradford method (1a). The purified capsids were analyzed in a Philips CM12 electron microscope after negative staining with 0.05 M uranyl acetate (pH 3.6). The purity was checked in sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Production of MAbs. The production of PAR1 (36), PAR3 (36), and BE11 (24) has been described. The antigen used was B19 virus isolated from viremic serum, and antibodies were screened by an ELISA (PAR1 and PAR3) and a dot blot ELISA (BE11), using B19 virus as the antigen. For these three MAbs, ascites fluid was obtained; in the case of PAR1, this material had been protein A-column purified.

MAbs D1 to D15 were produced by injecting two 8-week-old BALB/c mice intraperitoneally with 10 µg of purified VP2 capsids in Freund's incomplete adjuvant on days 0 and 14. The mice were boosted again on days 28 and 42 with 20 µg of VP2 in Freund's incomplete adjuvant. The spleens were removed, and two separate fusions were made with SP2/0-Ag8 myeloma cells, using polyethylene glycol 4000. The hybridoma cells were examined for the production of anti-B19 virus MAbs by an ELISA, using purified VP2 capsids coated on the microtiter plates. Positive clones were recloned twice, and hybridoma supernatants were used for testing. MAbs 3A2 and 3B2 were the gift of Jaap Middeldorp (Organon) and were produced by using a bacterial B19 virus-β-galactosidase fusion protein which contains a truncated VP1 protein up to nucleotide residue 4619 (28). Protein A-column-purified ascites fluid was obtained for testing.

Construction of pGEX expression plasmids. Plasmid DNA manipulations were performed as described previously (18). Plasmid transformations were carried out in *Escherichia coli* pC2495 (a derivative of JM101 obtained from Phabagen, Utrecht, The Netherlands). Restriction enzymes, the Klenow large fragment of DNA polymerase, T4 DNA ligase, and deoxynucleoside triphosphates were obtained from Pharmacia. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. The pGEX plasmid expression vectors pGEX-2T and pGEX-3X (described in reference 29) were obtained from Pharmacia. They direct the synthesis of foreign polypeptides in *E. coli* as fusions with the C terminus of a 26-kDa glutathione S-transferase (GST) from the helminth *Schistosoma japonicum*, under the control of an isopropylthiogalactopyranoside (IPTG)-inducible *tac* promoter. A polylinker that replaces the termination codon of Sj26 contains unique *Bam*HI, *Sma*I, and *Eco*RI recognition sites followed by TGA translation termination codons in all three reading frames. pGEX-2T contains the cleavage recognition sequence of the protease thrombin, and pGEX-3X contains that of factor X, which increases the molecular weight of GST to 275,000. These sites can be used to cleave the expressed polypeptide from GST after purification. pGEX-3X has a shift in the reading frame of the polylinker of +1 compared with pGEX-2T, and both vectors were used to obtain all B19 virus VP2 fusion proteins in frame with GST.

Seven pGEX clones expressing overlapping polypeptides of B19 virus VP1, designated VPG1 to VPG7, were generated. The source of B19 virus DNA, the isolation of which has been described previously (3), was a 2.49-kb *Hind*III-*Sca*I fragment between nucleotides 2430 and 4920 containing the complete coding region for VP1 (all nucleotide numberings are as described in reference 27) which had been ligated in the *Hind*III-*Sma*I sites of pUC19. For the expression of fusion proteins VPG1, VPG2, VPG4, VPG6, and VPG7, the respective fragments were made blunt (when necessary) and ligated in the *Sma*I site of pGEX-3X. The fragments encod-

ing the fusion proteins were as follows: VPG1, a 510-bp *Sau*3A-*Pvu*II fragment between nucleotides 2570 and 3050; VPG2, an 820-bp *Hind*II fragment between nucleotides 2880 and 3700; VPG4, a 510-bp *Nar*I-*Asp* 718 fragment between nucleotides 3570 and 4080; VPG6, a 580-bp *Sau*3A-*Eco*RI fragment between nucleotides 4340 and 4920; and VPG7, a 280-bp *Hinf*I-*Eco*RI fragment between nucleotides 4640 and 4920 (for VPG6 and VPG7, the *Eco*RI site originates from the pUC19 polylinker, as the *Sma*I site has been destroyed). For the expression of VPG3, a 790-bp *Hae*III fragment between nucleotides 3210 and 4000 was isolated and ligated in the blunt-ended *Eco*RI site of pGEX-3X. For the expression of VPG5, a 500-bp *Bam*HI-*Pvu*II fragment between nucleotides 3900 and 4400 was ligated in the *Bam*HI-*Sma*I sites of pGEX-2T. After transformation, clones containing the correct inserts were selected by restriction enzyme analysis.

SDS-PAGE analysis of proteins. *S. frugiperda* cells were infected with the recombinant baculovirus expressing both VP1 and VP2 as described above, and total proteins in 2×10^4 cells were analyzed. For the pGEX fusion proteins, SDS-polyacrylamide gel electrophoresis (PAGE) analysis was used to screen for the correct orientation of the inserted VP1 DNA fragment. Fragments in the opposite orientation produce only GST, while those in the correct orientation produce fusion proteins of various sizes which are larger than the GST control. Individual colonies from the various clones were grown overnight at 37°C in Luria broth containing 25 µg of ampicillin per ml. These fresh cultures were diluted 1:10 in Luria broth containing ampicillin and grown for 1 h at 37°C before addition of IPTG to 0.1 mM. Incubation was continued for 3 h, and the bacteria from 1 ml of culture were pelleted and taken up in 40 µl of SDS sample buffer (consisting of 2.5% SDS, 5% glycerol, 2.5% 2-mercaptoethanol, 62.5 mM Tris-Cl, and 0.05% bromophenol blue [pH 6.8]). Ten-microliter amounts were analyzed in SDS-10% polyacrylamide gels (17) stained with fast green (Fig. 1B).

ELISA. Twenty-nanogram amounts of VP2 and VP1/2 capsids were coated in PBS (pH 7.4) overnight at 4°C onto microtiter plates (Polysorp; Nunc). Plates were washed with PBS containing 0.05% Tween 20 and incubated for 1 h at 37°C with the MAbs diluted 1:10,000 (PAR1, PAR3, and BE11), 1:1,000 (D1 to D9 and 3A2), or 1:100 (D10 to D15) in PBS (pH 7.4) containing 2% fetal calf serum, 0.05% Tween 20, and 0.01% methiolate (PFTM). The plates were washed and incubated for 30 min at 37°C with a 1:4,000 dilution in PFTM of peroxidase-labelled rabbit anti-mouse immunoglobulin (Ig) G (DAKO A/S, Glostrup, Denmark). After washing, the substrate *o*-phenylenediamine (Abbott) was added, and the mixture was incubated at room temperature for 30 min. The reaction was stopped with 4 N H₂SO₄, and the A_{492} was read.

RIA. The radioimmunoassay (RIA) was performed in duplicate as an IgM capture assay as described previously (8). After every step, plates were washed with PBS containing 0.05% Tween 20 (PBST), and the sera, B19 virus, and MAbs were diluted in PBST containing 10% fetal calf serum. Microtiter plates were coated overnight at 4°C with goat anti-human μ -chain serum (TAGO) in 0.05 M sodium bicarbonate buffer (pH 9.6). The plates were washed, 100 U of B19 virus-specific IgM (as determined in the RIA) was added, and the plates were incubated for 3 h at 37°C. The plates were again washed, 0.2 ml of B19 virus antigen (isolated from the plasma of a viremic donor) or control antigen (prepared from the plasma of a nonviremic donor)

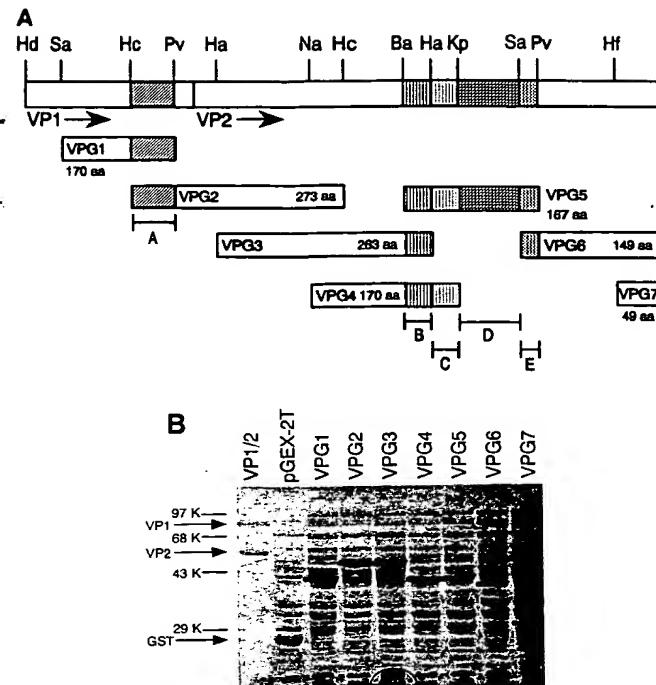


FIG. 1. (A) Positions of the pGEX fusion proteins VPG1 to VPG7 with respect to VP1 and regions to which MAbs bind; (B) SDS-polyacrylamide gel analysis of fusion proteins and baculovirus-produced VP1 and VP2. In panel A, the region to which MAbs 3A2 and 3B2 bind is designated A, the region to which MAbs D11 and D12 bind is designated B, the region to which MAb PAR3 binds is designated C, the region to which MAbs PAR1, BE11, D1 to D5, and D8 to D10 bind is designated D, and the region to which MAbs D6 and D7 bind is designated E. Restriction sites: Ba, *Bam*H; Ha, *Hae*III; Hc, *Hinc*II; Hd, *Hind*III; Hf, *Hinf*; Kp, *Kpn*I; Na, *Nar*I; Pv, *Pvu*II; Sa, *Sau*3A. aa, amino acids. In panel B, the positions of GST, VP1, and VP2 are indicated, and circles indicate positions of the fusion proteins.

was added to each well, and the plates were incubated for 3 h at 37°C. After washing, 0.2 ml of the MAb to be tested at 1:1,000 and 1:10,000 dilutions was added, and the plates were incubated overnight at 4°C. The plates were washed, 0.2 ml of ¹²⁵I-labelled sheep anti-mouse Ig (Amersham) was added, the plates were incubated for 2 h at 37°C and washed, and bound radioactivity was measured.

Western immunoblot analysis. Total proteins in insect cells expressing both VP1 and VP2 and in bacteria expressing the pGEX-VP1 fusion proteins were run in SDS-polyacrylamide gels as described above. The proteins were transferred onto a Zeta-probe nylon membrane (Bio-Rad), and the reactions were performed as described previously (3). The conjugate was alkaline phosphatase-labelled goat anti-mouse Ig (Promega). The blots were probed with the MAbs at the following dilutions: PAR1, PAR3, BE11, 3A2, and 3B2, 1:1,000; D1 to D9, 1:100; and D10 to D15, 1:50.

PEPSCAN. Overlapping nonapeptides covering amino acids 292 to 426 of the VP2 protein were synthesized and tested as described previously (11, 12). The first nonapeptide consisted of residues 292 to 300, the second consisted of residues 293 to 301, and so on. All peptides were tested against the MAbs in an ELISA. The ELISAs were repeated for those MAbs that gave a positive signal. Optical density values were read at 450 nm.

RESULTS

Reactivities of MAbs with native and recombinant parvovirus B19 particles. The reactivities of a panel of 19 MAbs were tested with baculovirus-produced B19 virus capsid proteins and native virus, using an ELISA and an RIA (Table 1). MAbs PAR1, PAR3, and BE11, which were raised against native B19, and D1 to D15, which were raised against baculovirus-produced VP2 capsids, reacted with the VP2 and VP1/2 capsids in the ELISA. These MAbs therefore recognize VP2-specific determinants, which is expected for D1 to D15 since these MAbs were raised against capsids consisting of VP2 alone. MAbs 3A2 and 3B2, which were raised against a bacterial β-galactosidase-VP1 fusion protein, reacted with the VP1/2 capsids in the ELISA and not the VP2 capsids and are therefore VP1 specific, as also shown by Western blot analysis (3) (Fig. 2).

The reactivities of the MAbs with native B19 virus in an RIA were tested to determine whether the determinants recognized by the MAbs made against the VP2 capsids are also found on the native virus. Of the 15 VP2-specific MAbs, D1 to D5 and D8 gave a positive reaction (D1 to D4 at a 1:10,000 dilution and D5 and D8 at a 1:1,000 dilution). Of the three MAbs that were raised against native virus, only PAR1 (at a 1:1,000 dilution) reacted with the B19 virus isolate used in the RIA.

Antigenicity of bacterial fusion proteins. The binding domains of the MAbs were localized by testing their reactivities with pGEX-GST fusion proteins in Western blots. Seven overlapping fusion proteins (VPG1 to VPG7) spanning the complete VP1 protein (Fig. 1A) were expressed in bacteria by cloning fragments of VP1 DNA at existing restriction sites in the pGEX expression vectors pGEX-2T and pGEX-3X. The fusion proteins produced are shown in Fig. 1B. It can be seen that the different proteins are produced in different amounts. The VPG7 fusion protein cannot be distinguished, and its presence and size were confirmed by affinity chromatography purification on immobilized glutathione (29). VPG1 and VPG5 could also be purified in this way, suggesting that these three polypeptides represent soluble areas of the VP1 protein (data not shown). Each MAb was tested in Western blots with baculovirus-expressed VP1 and VP2, pGEX-2T (as a control for reactivity with GST), and the fusion proteins (Table 1). None of the MAbs showed any reactivity with GST or any other bacterial proteins, confirming the specificity of the Western blots. 3A2 and 3B2 reacted only with VP1 in a Western blot and were therefore tested only with VPG1 and VPG2, since these fusion proteins cover the N-terminal unique part of VP1. They reacted with both VPG1 and VPG2, which localized the region to which they bind to a stretch of 57 amino acids (region A in Fig. 1A). The reactivity of 3A2 is shown in Fig. 2A.

The rest of the MAbs (except D13 to D15) reacted with both VP1 and VP2 in the Western blot, confirming the results from the ELISA showing that they recognize determinants on VP2. D13 to D15 reacted with the capsids in the ELISA and probably recognize conformational epitopes. Of the MAbs that reacted with VP1 and VP2 in the Western blot, D11 and D12 reacted with VPG3 to VPG5, which localized their binding site to a stretch of 33 amino acids (region B in Fig. 1A). The reactivity of D12 is shown in Fig. 2B. PAR3 reacted with VPG4 and VPG5, which localized its binding site to a stretch of 28 amino acids (Fig. 1A, region C). Its reactivity is shown in Fig. 2C. Ten MAbs (PAR1, BE11, D1 to D5, and D8 to D10) reacted with VPG5 alone in the

TABLE 1. Reactivities of MAbs with parvovirus B19 antigens

MAb	Binding domain ^a	Reactivity									RIA	
		ELISA			Western blot							
		VP2	VP1/2	VP1/2	1	2	3	4	5	6	7	
PAR1	D	2.861	2.329	+	-	-	-	-	+	-	-	+
PAR3	C	2.811	2.363	+	-	-	-	+	+	-	-	-
BE11	D	>2.990	2.827	+	NT ^c	-	-	-	+	-	-	-
D1	D	1.862	1.602	+	NT	-	-	-	+	-	-	+
D2	D	1.386	1.363	+	NT	-	-	-	+	-	-	+
D3	D	1.905	1.636	+	NT	-	-	-	+	-	-	+
D4	D	2.537	2.069	+	NT	-	-	-	+	-	-	+
D5	D	0.680	0.489	+	NT	-	-	-	+	-	-	+
D6	E	0.436	0.252	+	-	-	-	-	+	+	-	-
D7	E	0.782	0.398	+	-	-	-	-	+	+	-	-
D8	D	0.624	0.421	+	NT	-	-	-	+	-	-	-
D9	D	0.359	0.216	+	NT	-	-	-	+	-	-	-
D10	D	1.005	0.617	+	-	-	-	-	+	-	-	-
D11	B	>2.990	>2.990	+	-	-	+	+	+	-	-	-
D12	B	2.740	2.121	+	-	-	+	+	+	-	-	-
D13		0.932	0.800	-	NT	NT	NT	-	-	-	NT	-
D14		0.716	0.471	-	NT	NT	NT	-	-	-	NT	-
D15		1.326	1.101	-	NT	NT	NT	-	-	-	NT	-
3A2 ^d	A	0.046	0.475	+	+	+	NT	NT	NT	NT	NT	-
3B2 ^d	A	NT	NT	+	+	+	NT	NT	NT	NT	NT	NT

^a As shown in Fig. 1A.^b 1 to 7 represent VPG1 to VPG7.^c NT, not tested.^d Did not react with VP2 in the Western blot.

Western blot, which localized their binding sites to a stretch of 84 amino acids shown as region D in Fig. 1A. The reactivity of PAR1 is shown in Fig. 2D. Lastly, D6 and D7 reacted with VPG5 and VPG6, which cover a 22-amino-acid stretch (region E in Fig. 1A). The reactivity of D6 is shown in Fig. 2E. Since this MAb reacted strongly with VPG5 and weakly with VPG6, just before development of the color reaction, the filter was cut in strips to separate the lanes containing VPG5 and VPG6 from the rest of the samples. In repeat experiments, in which the filters were intact and thus developed to the same extent, the reactivity of D6 remained specific for the two fusion proteins. The same was true for D7. The extra bands seen in the Western blots are due to breakdown products.

PEPSCAN analysis. To precisely map the binding sites of the MAbs that bind to regions C, D, and E, a PEPSCAN was performed. Overlapping nonapeptides covering amino acids 292 to 426 of the VP2 protein were synthesized and tested as described above. Three MAbs reacted positively (Fig. 3). The binding site of D2 covered six peptides and was localized to a stretch of 14 amino acids between residues 321 and 334 of VP2. D10 bound to six peptides covering the 14 amino acids between 347 and 360, and PAR1 bound to eight peptides covering amino acids 354 to 369, a stretch of 16 amino acids. 3A2, which served as a negative control, did not react with any of the peptides analyzed in the PEPSCAN. None of the remaining MAbs tested (PAR3, BE11, D1, and D3 to D9) gave a signal in the PEPSCAN ELISA, which suggests that their binding sites are longer than 9 amino acids (BE11 has been previously mapped to a peptide of 17 amino acids [24]) or that some degree of conformation is involved in the recognition site.

DISCUSSION

Binding sites of the MAbs and localization of an immunodominant domain on the VP2 capsids. A panel of 20 MAbs was used to map epitopes on recombinant parvovirus B19 virus VP2 particles. The VP2 specificities of the 15 MAbs made against the VP2 capsids (D1 to D15) were confirmed by testing their reactivities in an ELISA with purified baculovirus-produced VP2 and VP1/2 capsids. The three MAbs made against the native B19 virus (PAR1, PAR3, and BE11) were also shown to recognize epitopes on VP2 in this way. The two MAbs (3A2 and 3B2) that did not react with VP2 in the ELISA were VP1 specific, as shown by Western blot analysis. Of the 15 MAbs raised against recombinant VP2 particles, 12 had sequential binding domains concentrated within a 167-amino-acid stretch of the VP2 protein between amino acids 259 and 426, as shown by their reactivities with bacterial fusion proteins expressing overlapping fragments of VP1. This finding suggests that this is an important immunogenic region of the recombinant VP2 capsids. The three MAbs (D13 to D15) that reacted with the VP2 and VP1/2 capsids in the ELISA but not with the same antigens in a Western blot probably recognize conformational epitopes. Within this 167-amino-acid stretch, the binding sites of four MAbs (D11, D12, D6, and D7) were mapped to discrete peptide fragments ranging from 22 to 33 amino acids (domains B and E; Fig. 1A). Domain D, an 84-amino-acid stretch within the immunogenic region, was the binding domain for eight of the MAbs (D1 to D5 and D8 to D10), which suggests it is the most important antigenic domain within this 167-amino-acid region. The three MAbs raised against native B19 virus also recognize epitopes within the 167-amino-acid region. Three MAbs, D2, D10, and PAR1,

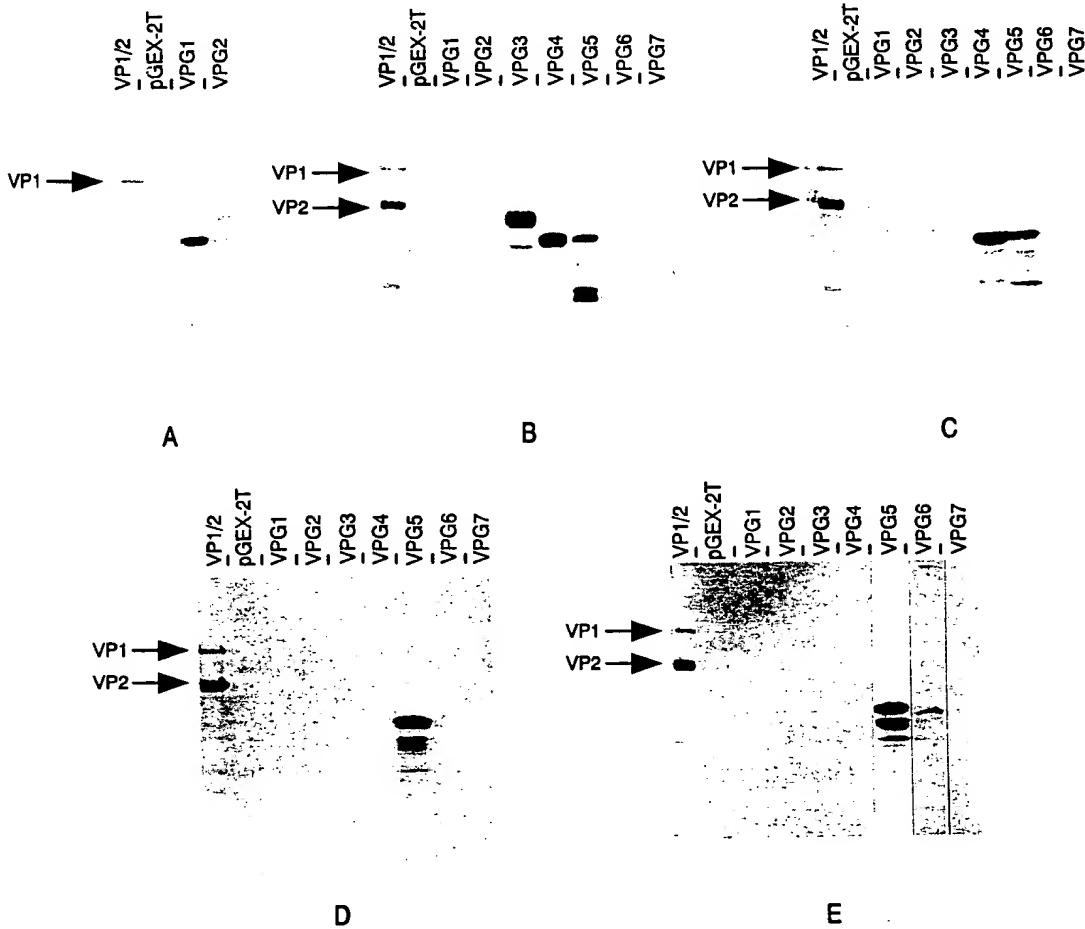


FIG. 2. Western blots showing reactivities of the MAbs with VP1 and VP2 produced in insect cells and the pGEX fusion proteins. (A) Reactivity of 3A2 at a 1:1,000 dilution; (B) reactivity of D12 at a 1:50 dilution; (C) reactivity of PAR3 at a 1:1,000 dilution; (D) reactivity of PAR1 at a 1:1,000 dilution; (E) reactivity of D6 at a 1:50 dilution (the binding patterns shown correspond to binding regions A to E in Fig. 1A).

could be fine-mapped within domain D by using a PEPSCAN (Fig. 3 and 4). An analysis of the peptides recognized by D2 and PAR1 made it possible to identify a core sequence of six amino acids (ISLRPG for D2 and TTYGNA for PAR1) that may be the essential residues for binding (Fig. 3, boxed residues). The binding site of D10 is a little more complex. The core sequence ISHG (residues boxed by a continuous line in Fig. 3) is present in all peptides which give a signal in the PEPSCAN ELISA. In addition, two residues at the extreme ends of the first and last positive peptides (TG and YG, respectively) are responsible for a significant increase in signal (residues boxed by dotted lines in Fig. 3). The presence of either of these end residues may result in the peptide adopting a conformation that enhances binding of the antibody.

The immunodominant domain on the VP2 capsids correlates with an important surface region on the native B19 virion. Evidence that the 167-amino-acid region on the recombinant VP2 capsids is also an important antigenic domain of the native B19 virion comes from a previous study in which peptides from hydrophilic regions of the VP2 protein were synthesized. These peptides were used to purify peptide-specific antibodies from human sera containing B19 virus-

specific IgG, and the antibodies were subsequently tested for the ability to neutralize and immunoprecipitate native virus (25). Five of these epitopes map to the antigenic region defined on the recombinant VP2 capsids. Four (S253, S309, S325, which is also the recognition site for BE11, and S359) were recognized by human antibodies that can both neutralize and immunoprecipitate native virus, and one (S288) was recognized by antibodies that can immunoprecipitate native virus. This region is therefore assumed to be on the surface of the native virion. The positions of these epitopes in relation to the epitopes mapped on the recombinant VP2 capsids are shown in Fig. 4; there is a high degree of overlap except for D6 and D7. The pGEX fusion protein VPG5, encompassing binding domains B to E of the VP2 capsids, was shown to be soluble, and therefore hydrophilic, by its purification by affinity chromatography on immobilized glutathione (29). Further, MAb PAR1 can immunoprecipitate native virions and MAb BE11 can neutralize virus infectivity, suggesting that they recognize surface epitopes. They both also recognize epitopes located in domain D of the antigenic region on the recombinant VP2 capsids. These lines of evidence indicate that this antigenic region is on the surface of the recombinant VP2 capsids.

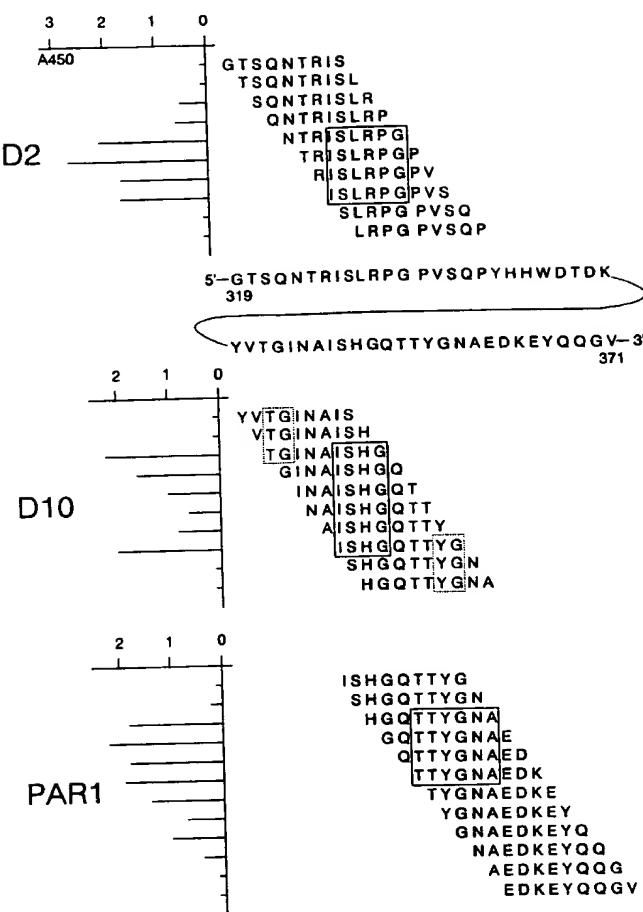


FIG. 3. PEPSCAN of amino acids 319 to 371 of VP2 reacted with MAbs D2, D10, and PAR1. The peptides and amino acids (boxes with solid and dotted lines) to which the three MAbs bind are shown. The vertical axis shows A_{450} values. D2 was tested at a 1:50 dilution, D10 was tested at a 1:2 dilution, and PAR1 was tested at a 1:1,500 dilution.

The immunodominant domain on the VP2 capsids correlates with the surface spike of CPV. In canine parvovirus (CPV), the only parvovirus for which the three-dimensional structure is known, a major part of the virion surface is made up

of the threefold spike which consists of four loops (32). By sequence comparison of the CPV and B19 virus VP2 proteins (using the University of Wisconsin Genetics Computer Group program), the positions of loops 3 and 4, the largest loops, were localized on the B19 virus VP2 protein; the positions of the residues situated in these loops are shown in Fig. 4. The epitopes mapped on the recombinant VP2 capsids, with the exception of D6, D7, and possibly D11 and D12, all lie between the projected surface loops. Additionally, the core sequence of the binding site of PAR1 (TTY GNA), which partially overlaps that of D10, was matched in the sequence comparison with CPV to amino acids K-387 to G-392, all of which (except T-388) are on the surface of the CPV virion (32). Also, it was possible to demonstrate direct binding of MAbs D2, D10, and PAR1 to the recombinant VP2 particles in the electron microscope, which confirms that the three epitopes are located on the surface of the particles (34). Thus, this important surface region of CPV and the native B19 virus corresponds to a major antigenic, probably surface, region of the recombinant VP2 capsids.

The VP2 capsids and native B19 virion share common epitopes. The recombinant VP2-specific MAbs were also tested with native B19 virus in an RIA, a capture system in which the virus should be more or less intact. Six of the MAbs, D1 to D5 and D8, which recognize a determinant in region D (Fig. 2), reacted, indicating that the synthetic and native particles share some common antigenic determinants. The three B19 virus-specific MAbs (PAR1, PAR3, and BE11) were also tested in the RIA, and only PAR1 reacted with the virus isolate (English) used. Since all three MAbs were raised against Japanese isolates, this finding suggests that there is antigenic variation between the different isolates. PAR1 recognizes a determinant common to the Japanese isolate, against which it was made, the English isolate of the RIA, and the recombinant VP2 and VP1/2 capsids which were derived from an independent Dutch virus isolate (3). PAR3 and BE11 both reacted with the VP2 and VP1/2 capsids in the ELISA; therefore, these particular determinants are common to the Japanese strains and the recombinant capsids derived from the Dutch virus isolate. Similarly, the VP2-specific MAbs (D6, D7, D9, and D10) that did not react in the RIA may well react with other isolates of B19 virus. Such variation between isolates has been shown only at the DNA level by restriction enzyme analysis (19, 20), but extensive sequence analysis of different isolates to detect amino acid substitutions has not been performed.

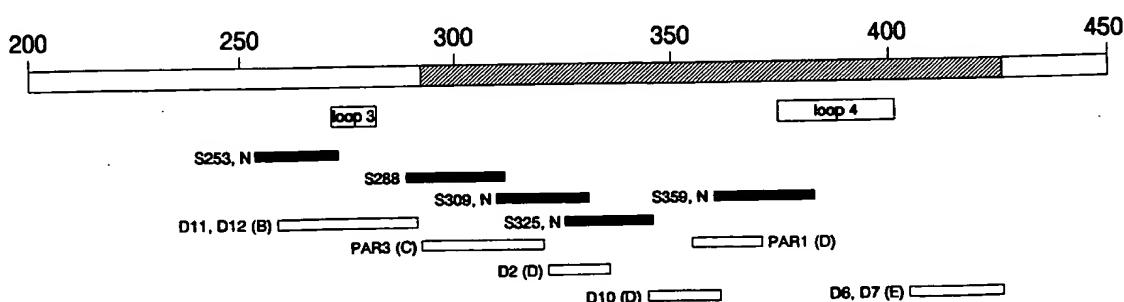


FIG. 4. Positions of the mapped determinants in relation to previously mapped B19 virus epitopes and the putative surface loops. The numbers 200 to 450 refer to VP2 amino acid residues; the shaded region is the area of the PEPSCAN; the two boxes show loops 3 and 4 extrapolated from the structure of CPV and constituting part of the spike region; thin black boxes represent epitopes mapped previously (25) and have the same nomenclature, N shows neutralizing epitopes (S253 is the determinant also recognized by BE11); thin white boxes represent epitopes mapped on the recombinant VP2 capsids, with names of the MAbs and the regions to which they bind as shown in Fig. 1A.

The fact that the VP2 (and VP1/2) capsids and native B19 virus have common antigenic domains and determinants, and possibly surface topology, is important if these capsids are to be used as a vaccine against B19 virus. In view of this consideration, the ability of the VP2 and VP1/2 capsids to induce neutralizing antibodies may need to be determined. Such a vaccine could be applied to specific high-risk patient groups, such as sufferers of sickle cell anemia and other types of hemolytic anemia in which a B19 virus infection can cause an aplastic crisis, and to prevent fetal death. Another possible use of the VP2 capsids is as a vaccine carrier for the presentation of foreign epitopes to the immune system, as has been described for hepatitis B virus core (7, 10, 30), and surface (22) antigens and yeast Ty virus-like particles (13). The recombinant VP2 capsids are immunogenic in both mice (this report) and rabbits, in which high-titered polyclonal sera have been produced (data not shown), and can be easily produced and purified in large amounts, approximately 10 mg/10⁹ cells. As for the positions of insertion of the foreign sequences, studies with hepatitis B virus core antigen have shown that insertion of epitopes in an immunodominant region of the particle, which is also surface exposed, renders these epitopes more immunogenic than the same epitopes fused to the N terminus (2, 26). For the VP2 capsids, for which the three-dimensional structure is not known, the well-defined sites recognized by MAbs D2, D10, and PAR1 may represent such immunoexposed determinants that would result in a high immunogenicity of inserted sequences.

ACKNOWLEDGMENTS

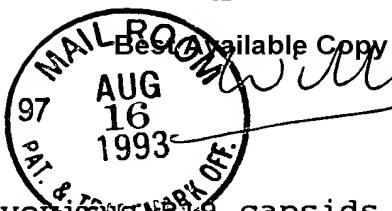
We thank Birgit Jacobsen for making the VP2 MAbs, Bernard Cohen for performing the RIA, Marcel Salimans and Mario van Bussel for practical assistance, and Neal Young for supplying the VP2 capsids for the electron microscope studies.

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IN PREPARATION

Chimeric parvovirus capsids for the presentation of foreign epitopes.

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Running head: Parvovirus capsids as vaccine carrier

EXHIBIT C
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Abstract

Chimeric proteins consisting of the VP2 capsid protein of human parvovirus B19 and defined linear epitopes from human herpes simplex virus type 1 and mouse hepatitis virus A59 inserted at the N-terminus and at a predicted surface region were expressed by recombinant baculoviruses. The chimeric proteins correctly expressed the inserted epitopes and assembled into empty capsids. Immunoelectron microscopy indicated that the epitopes inserted in the loop were exposed on the surface of the chimeric particles. The chimeric capsids were immunogenic in mice and antibodies specific for the inserted sequences were induced. In the case of MHV, antibodies were produced that recognized the epitope in the context of native virus. Mice immunized with the chimeric capsids were partially protected against a lethal challenge infection with live virus.

Introduction.

In the past few years, considerable interest has been focused on the use of polymeric particulate proteins for the presentation of epitopes or polypeptides as an improvement on peptide and subunit vaccines (reviewed in Kingsman and Kingsman, 1988). These carrier molecules offer a safe alternative to inactivated whole virus and in some cases result in the same degree of protection against infection with live virus. Examples include hepatitis B virus core antigen particles (HBcAg) for the presentation of epitopes from human rhinovirus type 2 (Francis et al., 1990), protein gp41 of HIV-1 (Borisova et al., 1989) and a neutralizing epitope from the VP1 protein of foot and mouth disease virus (Clarke et al., 1987), hepatitis B virus surface antigen particles (HBsAg) for presentation of the repetitive epitope of the circumsporozoite protein of Plasmodium falciparum (Rutgers et al., 1988) and yeast Ty virus-like-particles (VLPs) for the presentation of the human immunodeficiency virus (HIV-1) V3 loop (Griffiths et al., 1991). The particles have been produced in large amounts in bacterial, yeast and insect cell expression systems, are non-infectious and their particulate structure may make it possible to immunize in the absence of adjuvant.

We describe here a new carrier particle for the insertion and presentation of foreign epitopes, based on the major structural protein (VP2) of human parvovirus B19. This 58 kDa protein assembles into capsids resembling the native B19 virion when expressed from a baculovirus recombinant in insect cells (Brown et al., 1991b; Kajigaya et al., 1991). The particles can easily be

produced in large amounts (10 mg per 10^9 cells, Brown et al., 1991b) and have been shown to be immunogenic in mice (Brown et al., 1992) and rabbits (data not shown). Capsids incorporating approximately six copies of VP1 (the minor capsid protein) per particle could also be expressed (Brown et al., 1991b) and as the two proteins differ only in that VP1 has an extra N-terminal 227 amino acids, it was assumed that addition of a short stretch of foreign amino acids to the VP2 particle would not disrupt the particle structure.

In this report, we investigate whether the VP2 particles can incorporate and correctly express foreign sequences at different positions while maintaining their structure, whether the inserted sequences are immunogenic in the context of the chimeric particle and protect against a challenge infection, and whether immunogenicity or the level of protection are influenced by the position of insertion.

Since neither the three-dimensional (3-D) structure of the VP2 particles nor the sites of antigenic determinants were known at the time of these experiments, the epitopes were inserted at the extreme N-terminus of the VP2 gene by analogy to HBcAg (Clarke et al., 1987) and at a postulated surface loop on the particle. This loop was localized by sequence comparison with the VP2 protein of canine parvovirus (CPV) for which the 3-D structure is known (Tsao et al., 1991). The insertion of sequences into such surface loops should not disrupt particle assembly.

As a challenge model, two viruses were chosen for study, the human

herpes simplex virus 1 (HSV-1) and murine hepatitis virus (MHV), strain A59, both of which cause disease in mice. HSV-1 causes viral encephalitis and epitope VII consisting of amino acids 9-21 of the envelope glycoprotein gD was used for insertion in the VP2 capsids. This epitope is common to the gD protein of both HSV-1 and HSV-2 and produces virus neutralizing antibodies (Cohen et al., 1984). MHV-A59 causes hepatitis and either an acute encephalitis and/or a chronic progressive (subacute) demyelinating disease (Koolen et al, 1983). An epitope designated site A of the spike (S) protein of strain A59 (Talbot and Buchmeier, 1985) was used for insertion in the VP2 capsids. Both epitopes have been shown to protect against lethal challenge in the form of a peptide (Koolen et al., 1990; Geerligs et al., 1990b).

Materials and Methods.

DNA manipulations.

Plasmid DNA manipulations were performed as described (Maniatis et al., 1982). Plasmid and M13 transformations were carried out in pc2495 bacteria (a derivative of JM101 obtained from Phabagen, Utrecht) and M13 phage DNA containing uracil was prepared using E. coli CJ236 (dut ung F') (Kunkel et al., 1987). Restriction enzymes, the Klenow large fragment of DNA polymerase, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase and dNTPs were obtained from Pharmacia. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim and uridine was obtained from Sigma. Sequencing was performed with a Pharmacia kit using standard M13 primers for single stranded templates and reverse primers (Promega) for double stranded plasmid templates.

Oligonucleotides.

The oligonucleotide for mutagenesis of the N-terminus of VP2 corresponding to residues 3130-3153 on the coding strand (residue numberings throughout the paper are according to the sequence of Shade et al., 1986) had the sequence 5'-TTCAGTTAGATCTGCAGAAGCCAG-3'. The *Bgl*III cloning site is underlined. The oligonucleotide for sequencing the VP2 gene at the site of epitope insertion in the loop corresponded to residues 3991-4005 of the noncoding strand and had the sequence 3'-CGGTCCCCGGTGATCA-5'. The oligonucleotides encoding the HSV gD epitope 9-21 and the MHV epitope binding site A of the S protein are shown in Fig. 1. Both have sticky ends compatible for insertion in *Bgl*III or *Bam*HI restriction sites.

Viruses and cells.

Autographa californica nuclear polyhedrosis virus (AcNPV), and recombinant baculoviruses were grown and plaque-assayed in monolayers of *Spodoptera frugiperda* (Sf) cells (Brown and Faulkner, 1977), obtained from the American Type Culture Center (CRL 1711). Cells were cultured in TC-100 medium (GIBCO/BRL) containing 10% fetal calf serum (FCS) and 50 mg/ml Gentamycin. All baculovirus handlings were performed according to Summers and Smith (1987). L-cells for the production of MHV strain A59 were grown in DMEM supplemented with 10% FCS and antibiotics. Infectious virus for the MHV challenge and for the ELISA was produced as described in Spaan et al. (1981). Vero cells for the production of HSV-1, strain McIntyre, were grown in medium 199 supplemented with 10% newborn calf serum and antibiotics. Monolayers were infected with HSV-1, strain McIntyre and infectious and inactivated virus was prepared as described previously (Geerligs et al., 1990b).

Plasmid constructs.

For the insertion of the HSV and MHV epitopes at the N-terminus of the B19 VP2 gene (Fig. 2), a *Bgl*II restriction site was created between nucleotides 3137-3142. The source of B19 DNA for cloning, the isolation of which has been described previously (Brown et al., 1990) was an almost full-length clone consisting of an *Aat*II fragment between nucleotides 102 and 5128 which had been made blunt and ligated in the *Hinc*II site of pUC7. From this the coding region of the B19 VP1 and VP2 genes was isolated as a 2.5 K *Hind*III/*Sca*I fragment (residues 2430-4920). Since the VP2 gene

already contains a *Bgl*II site at position 3944, the N-terminus of VP2 was isolated as a *Hpa*II/*Bam*HI 830 bp fragment upon which all further manipulations were carried out. The *Hpa*II site was filled in and the fragment was ligated in the *Hinc*II and *Bam*HI sites of M13mp18. Upon transformation, an M13 clone containing the correct insert was selected by restriction enzyme analysis of dsDNA. Site-directed mutagenesis (as described in Current Protocols in Molecular Biology, 1989) was performed on this clone. Individual phage plaques were picked and the presence of the *Bgl*II site was confirmed by sequencing. The correct M13 clone was *Hind*III/*Bam*HI digested and the 830 bp mutated N-terminal fragment was isolated from gel and ligated in the corresponding sites of pUC19. This construct (pB19NBg) was digested with *Bgl*III, dephosphorylated and ligated with the phosphorylated oligonucleotides encoding the HSV and MHV epitopes. Plasmids containing the MHV epitope were preselected by testing for the presence of a *Sca*I site and sequencing was performed to identify plasmids containing a single copy of the HSV epitope (pB19NH) and MHV epitope (pB19NM) in the correct orientation. These plasmids were digested with *Hind*III (in the polylinker of pUC19), the site was filled in and the N-terminal VP2 fragment was released by *Bam*HI digestion.

Subsequently, a three-point ligation was performed between this fragment, the C-terminal *Bam*HI/*Sca*I fragment of VP2 and *Hinc*II digested, dephosphorylated pUC7 vector. The resulting constructs, p7V2NH and p7V2NM, contained the whole VP2 gene with the MHV and HSV epitopes inserted five amino acids downstream of the N-terminal methionine of VP2.

The hybrid VP2 genes were isolated from gel after *Bam*HI partial

digestion and ligated in the dephosphorylated *Bam*HI site of the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987) behind the polyhedrin promoter. Plasmids containing a single insert were selected and the correct orientation of the hybrid VP2 genes with respect to the polyhedrin promoter was confirmed by restriction enzyme analysis. The constructs were designated pAcYV2NH and pAcYV2NM.

Epitope insertion in the loop.

The construct pAcB19VP2-YM1, consisting of the B19 VP2 gene cloned in the *Bam*HI site of pAcYM1 and described in Brown et al., 1990, was used for insertion in the *Bgl*II site in the loop at position 3944. The position of this loop was localized by sequence comparison with the CPV VP2 protein using the programme Compare from the University of Wisconsin Genetics Computer Group. This construct was digested with *Bgl*III, dephosphorylated and the HSV and MHV epitopes inserted as described above. Clones containing a single copy of the HSV and MHV epitope in the correct orientation, as determined by sequencing using the "loop" oligonucleotide, were designated pAcYV2LH and pAcYV2LM respectively and used for transfection.

Production of recombinant baculoviruses.

The four plasmid constructs were purified on CsCl gradients and 1 ug of DNA was cotransfected onto monolayers of 10^6 Sf cells with 100 ng of DNA from the recombinant baculovirus AcRP23.lacZ (the gift of Dr. R.D. Possee and described in Possee and Howard, 1987). This had been linearized at a unique *Bsu*36I restriction site

situated in the lacZ gene positioned behind the polyhedrin promoter. Transfections were performed using 10 μ l lipofectin (GIBCO/BRL) made up to 50 μ l H₂O and mixed gently with the DNA also in a 50 μ l volume. After a 15 minute incubation at room temperature, the mixture was added to the monolayer to which had been added 1 ml TC-100 (without FCS) after washing twice with the same medium. This was incubated overnight at 27°C after which 1 ml of TC-100 containing 10% FCS was added and the incubation continued for two days. Supernatants containing baculoviruses were plaque assayed and these were stained with an equal mixture of TC-100 containing 0.4% X-gal (Boehringer Mannheim) and a 0.05% solution of Neutral Red (Sigma) to aid identification of recombinant (white) plaques. Blue plaques were formed by undigested or religated AcRP23.lacZ DNA. Ten white plaques per construct were picked and dot-hybridization with a B19 VP2-specific probe was performed to identify recombinant viruses which were purified to homogeneity by three rounds of plaque purification. The recombinant baculoviruses were designated VP2.NH and VP2.LH for the HSV epitope inserted at the N-terminus and loop respectively, and likewise VP2.NM and VP2.LM for the MHV epitope.

Analysis and purification of chimeric proteins.

Sf cells were infected at an moi of 10 pfu/cell with recombinant baculovirus expressing the original VP2 protein (Brown et al., 1990) or the four hybrid VP2 proteins, AcNPV or mock-infected. Cells were harvested 48 h pi and total proteins from 5 \times 10⁴ cells were analyzed in 10% SDS-polyacrylamide gels (Laemmli, 1970). Samples were boiled for 5 min in SDS sample buffer (consisting of

2.5% SDS, 5% glycerol, 2.5% 2-mercaptoethanol, 62.5 mM Tris-Cl and 0.05% Bromophenol Blue, pH 6.8) before loading on gel and these were stained with Fast Green (Sigma). For Western blot analysis, performed as described in Brown et al. (1990), proteins from 5,000 cells were separated and transferred onto a Zeta-probe nylon membrane (BioRad). The blots were probed with MAbs PAR1 specific for VP2 (the gift of Dr. K. Sugamura and described in Yaegashi et al., 1989), MAbs EII specific for the HSV gD 9-21 epitope (the gift of Dr. A. J. Scheffer), MAbs 5B19.2 specific for the MHV epitope A (the gift of Dr. M. Buchmeier and described in Talbot and Buchmeier, 1985), and MAbs 3A2 specific for VP1 (Brown et al., 1990) as a negative control. The mAbs were diluted 1:1,000 and the conjugate was AP-labelled goat-anti-mouse IgG (Promega). To determine if the hybrid VP2 proteins expressed in insect cells could assemble into capsids, insect cells infected at moi 1 were harvested 72 h pi. The cells were lysed and the capsids purified as described in Brown et al. (1992). Proteins were analyzed by SDS-PAGE (3 µg amounts) and in Western blots (200 ng amounts) as described above. For the large scale production of proteins, infection of insect cells with the recombinant baculoviruses and purification of the capsids was as described above.

Electron microscopy of capsids.

Purified capsids were analyzed in a Philips CM12 electron microscope after negative staining with 0.05 M uranyl acetate (pH 3.6). For immunogold labelling of capsids, preparations were made on 150 mesh nickel grids covered with formvar and coated with a 3 nm carbon layer. Prior to use the grids were exposed to a glow

discharge in air for 10 s. Grids were incubated for 1 min on drops of capsid suspension, blocked for 20 min in PBS containing 1% BSA and incubated for 20-30 min with a 1:1000 dilution of the mAbs PAR1, EII and 5B19.2. The grids were washed with 25 drops of PBS before incubation for 20 min on a suspension of 7 nm protein A-gold (pAg-7, prepared as described in van Lent and Verduin, 1985) diluted to $A_{520} = 0.1$ in PBS containing 1% BSA. The grids were washed with 25 drops of PBS followed by 10 drops of distilled water and stained with 0.05 M uranyl acetate. Preparations were observed as above.

Synthetic peptides.

The peptide representing binding site A of the spike protein of MHV coupled to a T-helper determinant of the HA1 polypeptide of influenza virus (peptide E2HA) is described in Koolen et al. (1990). The HSV-1 peptides consisting of amino acids 7-21 (coupled to ovalbumin) and 9-21 of the gD protein (epitope VII) are described in Geerligs et al. (1990b).

Animal experiments.

Groups of four to six week old female BALB/c mice were injected intraperitoneally (HSV experiment) or subcutaneously (MHV experiment) with 20 μ g amounts of the capsid antigens VP2, VP2.NH, VP2.LH, VP2.NM and VP2.LM emulsified in complete Freund adjuvant. In the HSV experiment, mice were also injected with 100 μ g amounts of peptide 7-21 coupled to ovalbumin or 3.9×10^6 pfu of heat inactivated HSV-1. Control groups were injected with PBS. Three and six weeks later, the mice were boosted with antigen or PBS

emulsified in incomplete Freund adjuvant. In the HSV experiment, the mice were boosted a third time after 8 weeks and this time, the mice immunized with inactivated HSV-1 were boosted with 6.0×10^6 pfu of inactivated virus. The mice were challenged intraperitoneally 10 days after the last booster with 2.6×10^9 pfu of HSV-1 per animal. In the MHV experiment, the mice were intracerebrally challenged 30 days after the last booster with 10^4 pfu of wild type MHV-A59 per mouse. Sera were collected prior to the challenge infection, seven (HSV) or ten (MHV) days after the last booster immunization.

ELISA

Pre-challenge sera from the HSV experiment were tested in a twofold dilution series, starting at 1:200, for their ability to react with peptide 9-21 in an ELISA, performed as described by Geerligs et al. (1990a). Sera giving an extinction of more than 0.2 were considered to be positive (this cut-off value was obtained from previous experiments with polyclonal mouse sera either positive or negative for antibodies to peptide 9-21). Pre-challenge sera from the MHV experiment were tested in triplicate for their ability to react with peptide E2HA and with native MHV virus in an ELISA. For the peptide ELISA, 0.5 ug amounts of peptide were coated per well and for the ELISA with whole virus, approximately 5×10^4 pfu of PEG-precipitated MHV were coated per well in 50 mM sodium bicarbonate, pH 9.6. The coating was performed overnight at 4°C using microtiter plates (Nunc, Polysorp). Plates were washed with PBS containing 0.05% Tween-20, 2% FCS and 0.01% merthiolate (PFTM) and incubated for 1 h at 37°C

with sera diluted in PFTM. For the peptide ELISA, sera were tested in a tenfold dilution series, starting at a 1:100 dilution and ending at a 1:10,000 dilution. For the virus ELISA, sera were diluted 1:20, 1:100 and 1:1,000. The plates were washed and incubated for 30 min at 37°C with a 1:4,000 dilution of peroxidase-labelled goat-anti-mouse Ig (Dakopatts, Denmark). After washing, *o*-phenylenediamine substrate (Abbott) was added and incubated at rt for 30 min in the dark. The reaction was stopped with 4 N H₂SO₄ and absorbance values were read at 492 nm.

Results.

Production of baculovirus recombinants expressing chimeric proteins.

The cloning scheme for the insertion of the HSV and MHV epitopes (the sequences of which are shown in Fig. 1.) at the *N*-terminus of the VP2 gene and the production of the baculovirus recombinants is shown in Fig. 2. The position of the *Bgl*III site in the VP2 gene used for insertion of the epitopes in the loop is also shown. The presence of the hybrid VP2 genes in the plaque-purified recombinant baculoviruses was checked at the DNA level by restriction analysis (data not shown).

Characterization of the chimeric proteins.

Total proteins in insect cells infected with the recombinant baculoviruses were analysed in 10% SDS-polyacrylamide gels (Fig. 3A). This demonstrated the presence of a protein migrating at a slightly higher position compared to VP2 in the lanes containing proteins from the four recombinants VP2.NH, VP2.LH, VP2.NM and VP2.LM. The increased size was due to the peptide insertions of 13 (HSV) and 11 (MHV) amino acids. The identity of the chimeric proteins was confirmed by Western blot analysis with the VP2-specific MAb PAR1 and the presence, and correct expression, of the HSV and MHV epitopes was confirmed by Western blot analysis with MAb EII specific for the HSV epitope and MAb 5B19.2 specific for the MHV epitope (data not shown). None of the recombinant proteins reacted with a VP1-specific MAb.

Lysates of insect cells expressing the chimeric proteins were analyzed in CsCl gradients and in all four cases, an opalescent

band could be seen. The proteins in these bands were examined in SDS-polyacrylamide gels and in all cases, a predominant protein was seen that migrated at a slightly higher position compared to VP2 (Fig. 3B). The identity of the chimeric proteins and the correct expression of the HSV and MHV epitopes was again confirmed by Western blot analysis as described above and shown in Fig. 3C. All four chimeric proteins show a double band in the Western blot which may suggest slight instability compared to VP2. The purified proteins were examined in the electron microscope and all formed capsids with the same size and appearance as VP2 (Fig. 4 and Brown et al., 1991b). The amounts of chimeric particles produced were approximately equal to that for VP2 which yields up to 10 mg of purified particles per 10^9 cells (Brown et al., 1991b).

Immunological characterization of intact chimeric capsids.

To determine if the inserted epitopes were accessible on the surface of the capsids, immunogold labelling was performed on intact capsids with the epitope-specific MAbs EII and 5B19.2 and the VP2-specific MAb PAR1. The results were observed in the electron microscope and are shown in Fig. 4. In the case of the capsids VP2.NH and VP2.NM in which the epitopes had been inserted at the N-terminus, neither the HSV or MHV epitope-specific MAbs reacted. The chimeric particles VP2.LH and VP2.LM containing the epitopes in the loop were both labelled with the specific MAbs, with VP2.LH showing a higher degree of labelling than VP2.LM. The VP2 particles were poorly labelled by the VP2-specific MAb PAR1 (Fig. 4), as were the chimeric capsids (data not shown). Controls in which VP2 particles were tested with MAbs EII and 5B19.2,

VP2.NH/LH with MAb 5B19.2 and VP2.NM/LM with MAb EII showed no aspecific labelling. The control experiment with VP2.LH and 5B19.2 is shown in Fig. 4.

The two epitopes used in this study were also inserted at the C-terminus of the VP2 gene and baculovirus recombinants expressing the chimeric proteins were isolated. However, these chimeric proteins did not form particles and were not included in further experiments.

Immunogenicity of the chimeric capsids.

The ability of the chimeric proteins to induce an immune response directed against the inserted epitopes was examined by immunizing mice and testing sera in an ELISA using the peptide epitopes (9-21 for HSV and E2HA for MHV) as antigen. The results are shown in Table 1. Of the mice immunized with VP2.NH and VP2.LH, five in each group showed a positive antibody response to peptide 9-21. Seven of the mice immunized with the peptide epitope 7-21 coupled to ovalbumin showed a positive response while of the mice immunized with inactivated HSV-1, four showed a positive response with low titers. Of the mice immunized with VP2.NM, nine showed a positive antibody response to peptide E2HA while all ten mice immunized with VP2.LM showed a positive response. The anti-peptide titers from the mice immunized with VP2.NM were generally higher than from mice immunized with VP2.LM.

The ability of the chimeric capsids to produce sera in mice that recognize the epitope in the context of the native virus was tested in ELISAs using native virus as antigen and the results for

MHV are shown in Table 1. Nine mice immunized with VP2.NM and all ten mice immunized with VP2.LM reacted with MHV. Again, the titers from mice immunized with VP2.NM were generally higher. Sera from mice immunized with the VP2.NH and VP2.LH capsids and peptide 7-21 showed a low reactivity with HSV-1, with three from the VP2.NH group giving low titers, one from the VP2.LH group and two from the peptide 7-21 group. That the HSV-1 virus used in this ELISA was functional was demonstrated by the fact that sera from all mice immunized with inactivated HSV-1 reacted. None of the control sera from mice immunized with the VP2 capsids or PBS showed any reactivity with the peptides or viruses in the ELISAs (data not shown).

The sera were also tested in an ELISA using VP2 as antigen. All mice immunized with the VP2 capsids and the chimeric capsids showed a high antibody response to the VP2 capsids with absorbance values ranging from 1.288 to >2.99 at a 1:1,000 dilution of sera.

Protection of mice from a lethal challenge infection by vaccination with the chimeric capsids.

To determine if vaccinated mice were protected from an infection with live virus, mice were challenged with a lethal dose of HSV-1 (2.6×10^9 pfu per mouse) or MHV-A59 (10^4 pfu per mouse) (LD50: not known for HSV; MHV?) and the number of surviving mice is shown in Table 2. Immunization with the chimeric capsids VP2.NH gave a similar level of protection (70%) to immunization with peptide 7-21 coupled to ovalbumin (75%), even though the amount of peptide epitope in the capsids was 213 times less (0.47 μ g per immunization) than the amount of peptide 7-21 used (100 μ g per

immunization). Immunization with VP2.LH gave a lower level of protection (45%) than peptide 7-21. For both VP2.NH and VP2.LH, the level of protection was significantly higher than the VP2 control (11%). The highest level of protection (80%) was seen in mice immunized with inactivated HSV-1.

In the MHV experiment, immunization with the chimeric capsids VP2.NM and VP2.LM both gave a level of protection of 60%, which was significantly higher than the VP2 (0%) and PBS (10%) controls. Of the six mice that survived the challenge with MHV after immunization with VP2.NM, five showed pre-challenge antibody titers of 1,000 or above and one mouse showed a titer of 100 when tested with the E2HA peptide in an ELISA while the four mice that died had a titer of 100 or less. All six survivors had a titer of 1,000 when tested with native MHV in an ELISA as did three of the mice that died. Of the six mice immunized with VP2.LM, the only two mice that had an anti-peptide and anti-virus titer of 1,000 survived the challenge. Of the remaining four survivors, two showed titers of 100 in the E2HA ELISA and 1,000 in the MHV ELISA and two showed titers of 100 in both. All four mice that died showed titers of 100 in the E2HA ELISA and in the MHV ELISA, two showed titers of 1,000 and two showed titers of 100 or less.

Discussion.

- Parvovirus capsids can incorporate and express foreign epitopes at more than one location while retaining their structure.

We have examined the potential of baculovirus produced parvovirus B19 VP2 capsids to act as a carrier molecule for the presentation of foreign viral sequences and to protect mice from a lethal challenge infection. Since the position of insertion has been shown to be important for the immunogenicity of HBcAg (Brown et al., 1991a; Schodel et al., 1992) and HBsAg carrier complexes (Delpeyroux et al., 1990), the insertions of 13 (HSV gD epitope 9-21) or 11 (MHV spike protein epitope A) amino acids were made at the N-terminus and in a postulated surface loop of the VP2 protein. In both cases the chimeric proteins were able to assemble into particulate structures. The epitopes were correctly expressed, as determined by immunological analysis, and immunoelectron microscopy showed that insertion in the loop resulted in a greater surface accessibility of the epitopes than insertion at the N-terminus.

The chimeric capsids are immunogenic in mice.

Immunization of BALB/c mice with all four chimeric capsids (VP2.NH, VP2.LH, VP2.NM and VP2.LM) resulted in an immune response directed against the inserted HSV or MHV peptide epitope. This response was slightly higher in the mice immunized with the capsids in which the epitope had been inserted at the N-terminus than in the mice immunized with the capsids in which the epitope was inserted in the loop. Sera from mice immunized with the HSV peptide 7-21 showed a higher response to the peptide compared to

mice immunized with the chimeric capsids which may be due to a dosage effect (per immunization, the capsids contain 213 times less epitope than in peptide 7-21).

The mice immunized with VP2.NM and VP2.LM also developed an immune response to the native MHV virus indicating that presentation of epitope A of the spike protein at the N-terminus of the VP2 capsids and in an exposed loop induced antibodies that recognized the epitope in the context of the native virion. Sera from mice immunized with VP2.NH, VP2.LH and the peptide 7-21 showed a low response to the native HSV-1 virus in an ELISA (tested at a dilution of 200). This is in agreement with previous studies in which immunizations with peptide 9-21 carrier protein conjugates gave rise to low HSV titers (ranging from 6 to 28) in neutralization assays but provided protection against a lethal challenge (Weijer et al., 1988; Geerligs et al., 1990b). This is not unique for gD as immunizations with gG, one of the other HSV glycoproteins, protected against a lethal challenge in the presence of very low antibody titers, also obtained in neutralization assays (Ghiasi et al., 1992).

The VP2 and chimeric capsids VP2.NH, VP2.LH, VP2.NM and VP2.LM were able to induce a strong immune response against VP2 in all mice even in the absence of adjuvant (absorbance values in an ELISA ranged from 0.566 to >2.99 at a dilution of 1,000) which suggests a high degree of stability and immunogenicity of the capsids. Optimization of the insertion site for foreign epitopes may direct the immune response more towards the epitope and make

it possible to immunize in the absence of adjuvant or with an adjuvant that has been approved for human use.

The chimeric capsids protect against lethal challenge with virus.

Immunization of mice with the chimeric capsids VP2.NH gave a similar level of protection to lethal challenge with HSV-1 as mice immunized with peptide 7-21 coupled to ovalbumin (even though the capsids contained 213 times less epitope than the peptide antigen per immunization) and to mice immunized with inactivated HSV-1. In general, immunization with inactivated virus is expected to give the highest level of protection and this level of protection was almost equalled by immunization with VP2.NH. The level of protection afforded by the capsids VP2.LH was the lowest of the four immunized groups although it was still significantly higher than that seen for the controls.

In the MHV experiment, the level of protection (60%) was the same for both chimeric capsids VP2.NM and VP2.LM and was significantly higher than the control groups. The fact that a number of mice (three immunized with VP2.NM and four immunized with VP2.LM) responded to MHV but did not survive the challenge infection may be explained by a difference in antibody specificity as a result of the epitope assuming more than one conformation. Neither of the chimeric capsids VP2.NM or VP2.LM afforded complete protection while in a previous study in which mice were immunized with peptide E2HA, 93% of the mice were protected (Koolen et al., 1990). This could be due to the 125 fold difference in the amount of peptide epitope (0.397 µg in the capsids as opposed to 50 µg of E2HA per immunization) or the presence of the influenza virus T-

helper determinant in peptide E2HA. The latter is more likely as in the study of Koolen et al. (1990), immunization with peptide E2 coupled to KLH produced a much lower immune response to native MHV compared to E2HA, both in titer and protective ability, even though the same amounts of peptide epitope were used for immunizations. The superiority of the E2HA peptide may be explained by a better induction of memory cells (both B- and T-cells) due to the T-helper determinant which in turn resulted in a higher antibody response. Indeed, in the same study it was shown that the protective immune response following immunization with E2HA was dose dependent. These results corroborated previous findings which demonstrated that a 'foreign' helper T-cell determinant played an important role in the responsiveness to a peptide antigen of the VP1 protein of foot and mouth disease virus (Francis et al., 1987). In the case of the chimeric B19 capsids, addition of a T-helper cell determinant, specific for the inserted peptide epitope, may increase their ability to induce immunological memory and hence their protective properties. Since neither native B19 virus nor empty capsids (produced in Chinese hamster ovary cells) have been shown to stimulate T-cells from peripheral blood (Kurtzman et al., 1989), interference by T-cell determinants already present on the chimeric capsids should be minimal. Such interference was observed for HBcAg carrier particles where T-cells predominantly recognized the HBcAg carrier moiety and were therefore not primed against a T-cell site relevant to the pathogen and included in the inserted sequence (Schodel et al., 1992).

Influence of surface accessibility of the epitopes on immunogenicity and protection from infection.

In the experiments performed here, a greater surface accessibility of the HSV and MHV epitopes on the VP2.LH and VP2.LM capsids did not result in increased immunogenicity or protection. This was unexpected as results obtained with HBcAg showed that the immunological response to a human rhinovirus peptide incorporated in an immunodominant region of the particle was improved tenfold compared with amino terminal fusions (Brown et al., 1991a). We found that the immunogenicity of the capsids with the N-terminal insertions was slightly higher than that seen for the loop insertions and that the VP2.LH capsids showed a lower level of protection compared to VP2.NH. Recently, we have obtained evidence that the N-terminus of VP2 is situated on the surface of the capsids since antibodies directed against an N-terminal peptide react with intact particles (unpublished observations). However, it is possible that not all N-termini are surface-located and if this is the case, then the failure to detect the N-terminal insertions by immunogold labelling could be attributed to insufficient sensitivity. Surface location of (a portion of) the epitopes inserted at the N-termini of VP2 may explain the similar degree of immunogenicity and protection against infection afforded by the chimeric capsids. To definitively determine whether surface accessibility influences immunogenicity and the degree of protection, a dose titration with the chimeric capsids should be performed.

Finally, the optimal position for insertion of epitopes in the VP2

capsids should be determined as this may greatly influence the immunogenicity of the inserted sequences. It is expected that the parvovirus B19 particle will have the same β -barrel motif as CPV and as is observed in all other known icosehedral viruses that infect eukaryotes (Agbandje et al., 1991). In this case, two thirds of the B19 virion (and capsid) structure may be the result of insertions between the strands of the β -barrel which form primarily the surface features. These insertions would represent a large surface region for replacement by foreign sequences and expression at exposed loops may direct the response more towards the inserted epitope. As a first step in this direction, we have localized a large surface domain of 167 amino acids on the VP2 capsids and have fine-mapped three epitopes to four and six amino acids (Brown et al., 1992). These antigenic determinants may represent improved sites for epitope insertion. Furthermore, it may be possible to insert a large stretch of foreign sequences, or multiple copies of the same epitope, in place of the unique N-terminal region of VP1 in capsids consisting of both proteins (Brown et al., 1991b), especially as this region has been shown to be on the surface of recombinant VP1/2 capsids (Rosenfeld et al., 1992).

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Fig. 1. Oligonucleotides and amino acid sequences of the HSV gD epitope 9-21 (A) and the MHV spike protein epitope A (B) used for insertion in the VP2 capsid gene at the N-terminus (position 3137) and the loop (position 3944). Amino acids in bold comprise the epitopes. Underlined in (B) is the *Sca*I site.

Fig. 2. Cloning scheme for epitope insertion in the VP2 gene of parvovirus B19. Ba, *Bam*HI; Bg, *Bgl*III; Bs, *Bsu*36I; Hc, *Hinc*II; Hd, *Hind*III; Hp, *Hpa*II; Sc, *Sca*I; H, HSV-1 gD 9-21 epitope; M, MHV-A59 spike protein epitope A; N, N-terminus of B19 VP2; php, polyhedrin promoter; P, phosphate group; arrows show the direction of transcription.

Fig. 3. Analysis of chimeric proteins expressed in insect cells. Fast green stained SDS-polyacrylamide gels of total proteins in infected insect cells harvested 48 h postinfection (A) and of CsCl-gradient purified capsids (B). (C) Western blot analysis of purified capsids. PAR1, MAb specific for VP2; EII, MAb specific for HSV gD epitope 9-21; 5B19.2, MAb specific for MHV spike protein epitope A. Arrows at 58 kDa indicate the position of VP2 and the chimeric proteins; P, polyhedrin protein.

Fig. 4. Electron micrographs showing immunogold labelling of CsCl-gradient purified chimeric capsids with monoclonal antibodies. PAR1, MAb specific for VP2; EII, MAb specific for HSV gD epitope 9-21; 5B19.2, MAb specific for MHV spike protein epitope A. Bar represents 100 nm.

Table 1. Antibody titers obtained by immunizing mice with synthetic, biosynthetic and native virus antigens, as tested by enzyme-linked immunosorbent assay.

Antigen ^a / Antigen ^b	Positive antisera			Titer range
	HSV 9-21	E2HA	MHV	
VP2.NH	5/10	-	-	200-12,800
VP2.LH	5/11	-	-	100-3,200
HSV 7-21	7/8	-	-	400-51,200
HSV-1	4/10	-	-	200-400
VP2.NM	-	9/10	9/10	100-10,000 ^c
VP2.LM	-	10/10	9/10	20-1,000 ^d

^a Antigen used for immunization

^b Antigen used for ELISA

^c All positive sera in the MHV ELISA had titers of 1,000

^d In the E2HA ELISA the range was 100-1,000

Table 2. BALB/c mice surviving wild-type HSV-1 intraperitoneal challenge and wild-type MHV-A59 intracerebral challenge after immunizations.

HSV challenge		MHV challenge	
Antigen	Survivors	Antigen	Survivors
VP2	1/9 (11%)	VP2	0/10 (0%)
VP2.NH	7/10 (70%)	VP2.NM	6/10 (60%)
VP2.LH	5/11 (45%)	VP2.LM	6/10 (60%)
PEPTIDE 7-21	6/8 (75%)	PBS	1/10 (10%)
INACT. HSV-1	8/10 (80%)		
PBS	0/10 (0%)		

Fig. 1

A

S L K M A D P N R F R G K D
GA.TCT.CTC.AAG.ATG.GCC.GAC.CCC.AAT.CGC.TTT.CGC.GGC.AAA.GAC.G
A.GAG.TTC.TAC.CGG.CTG.GGG.TTA.GCG.AAA.GCG.CCG.TTT.CTG.CCT.AG

B

S L L G C I G S T C A E
GA.TCT.TTA.CTG.GGT.TGC.ATA.GGT.AGT.ACT.TGT.GCA.GAG.G
A.AAT.GAC.CCA.ACG.TAT.CCA.TCA.TGA.ACA.CGT.CTC.CCT.AG

Fig. 2

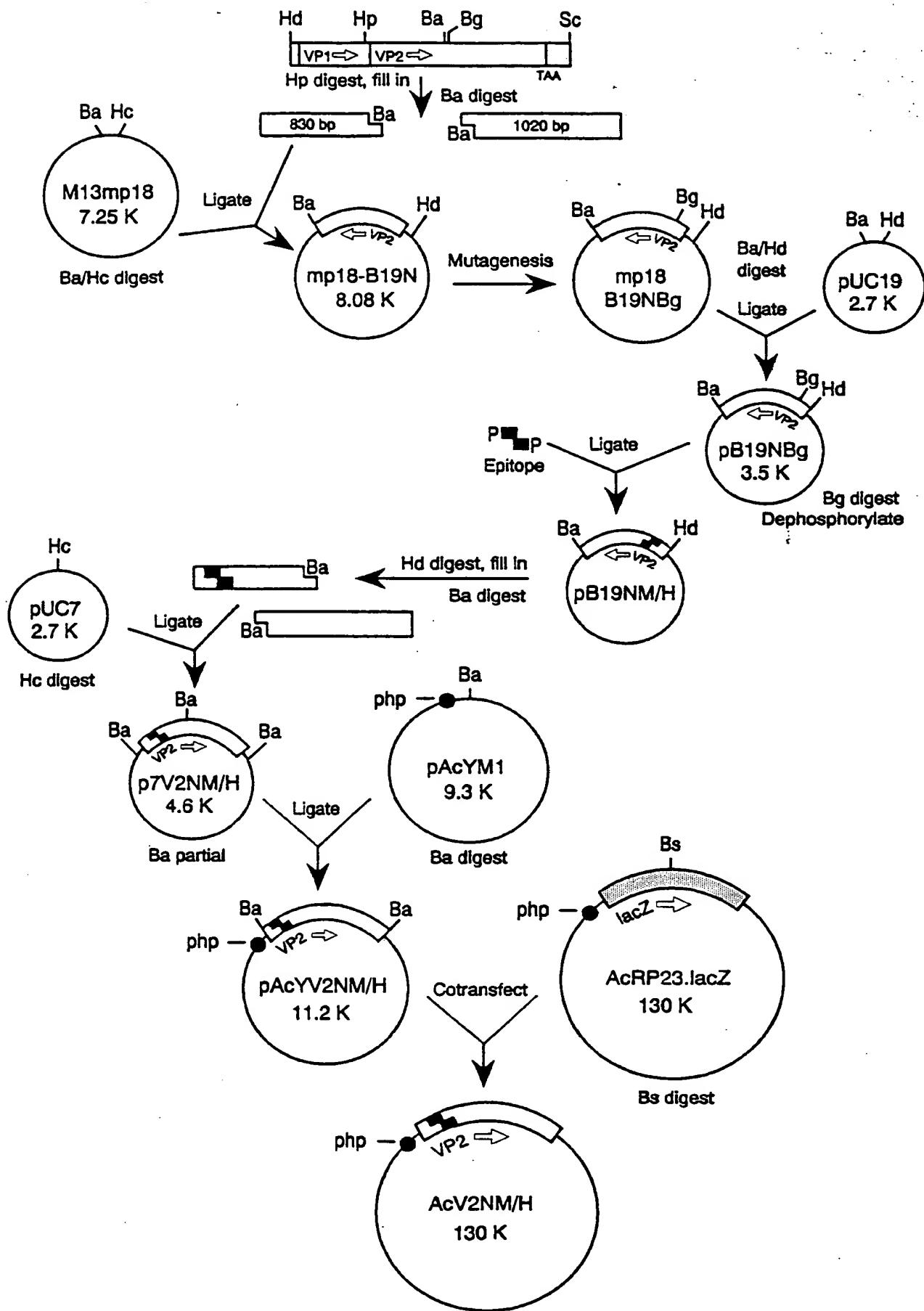
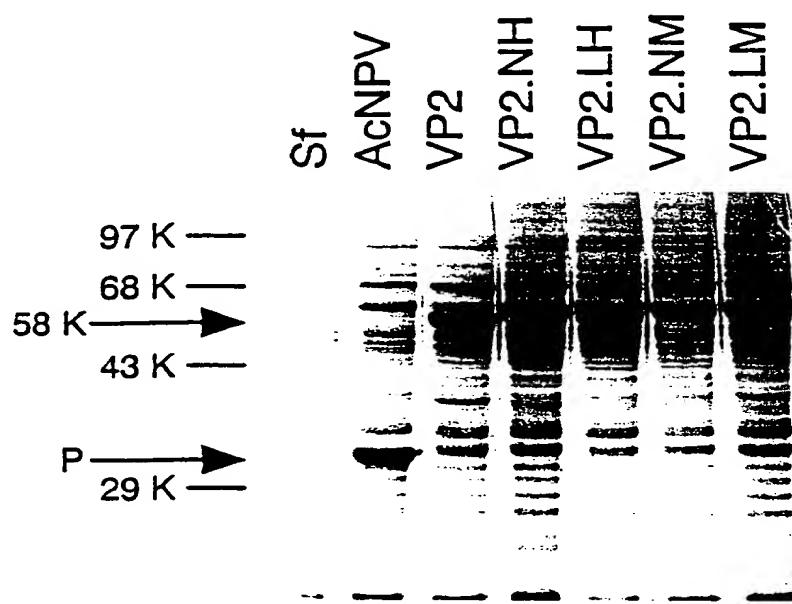


Fig 2

Fig. 3A

Best Available Copy



J.A. C.-B. Brown et al. / 701

(Printed copy)

Fig. 3B

Best Available Copy

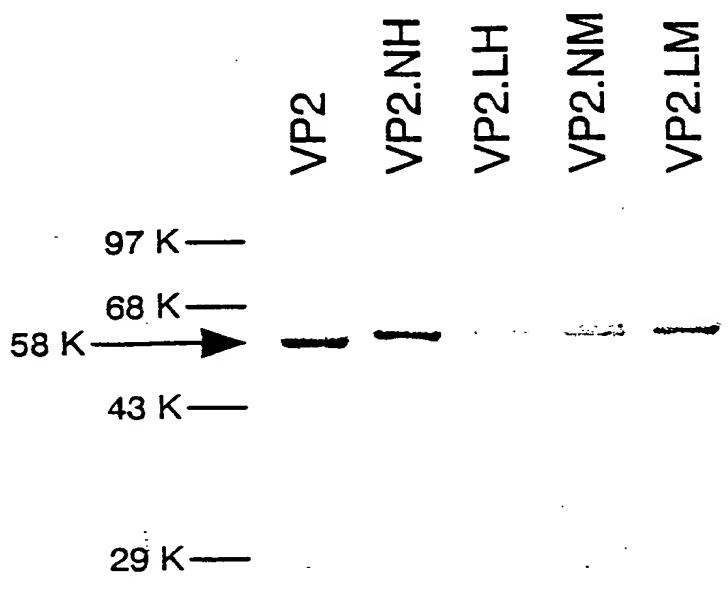


Fig. 3B CS Brown et al., Top ♀

Best Available Copy

VP2
VP2.NH
VP2.LH
VP2.NM
VP2.LM

58 K →

PAR1 EII 5B19.2

